

ABERRATIONS IN SERUM COMPLEMENT IN CHILDREN WITH OTITIS MEDIA

Complement components and C1q binding substances
in patients and experimental investigations on
complement activation and C1q binding by
bacterial constituents.

Karin Prellner

Lund 1981

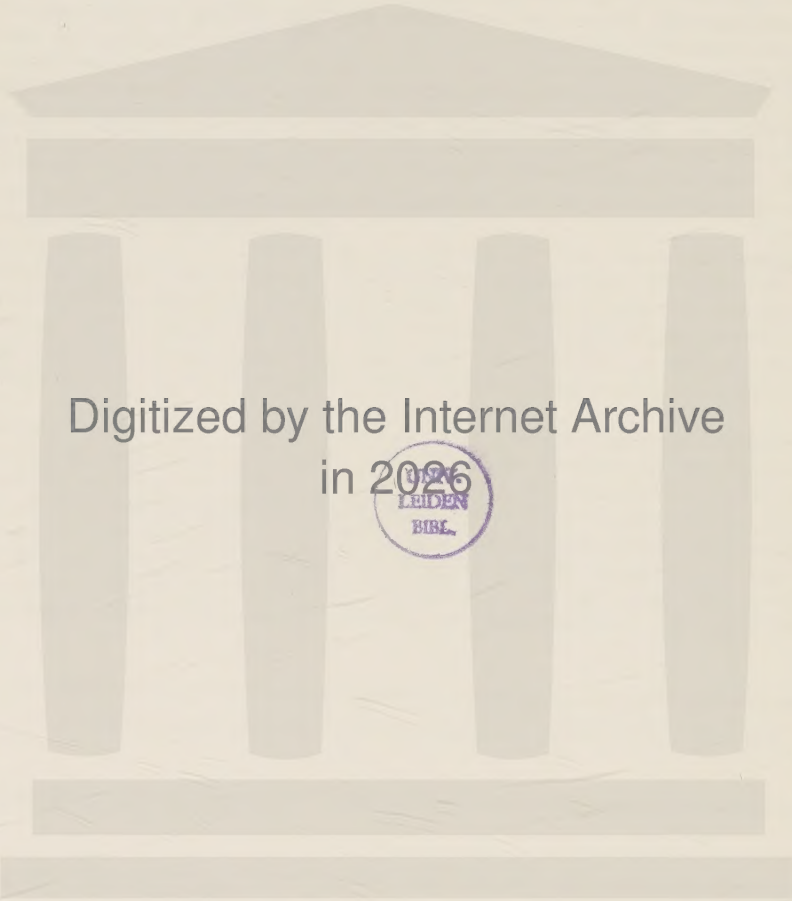
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To Thorbjörn, Susanne, Jonas and Liselott

The thesis is based on the following publications.
They will be referred to by their Roman numerals:

- I. Prellner, K. & Nilsson, N.I.: Complement aberrations in serum from children with otitis due to *S. pneumoniae* or *H. influenzae*.
Acta Otolaryngol (Stockholm). Accepted for publication.
- II. Laurell, A.-B., Nilsson, N.I. & Prellner, K.: Immune complexes and complement in serous and mucoid otitis media.
Acta Otolaryngol (Stockholm) 90, 290-296, 1980.
- III. Prellner, K.: Complement in pneumococcal infections with varying degrees of severity.
Scand J Infect Dis. In press.
- IV. Prellner, K.: Bacteria associated with acute otitis media have high C1q binding capacity.
Acta Pathol Microbiol Scand (C) 88, 187-190, 1980.
- V. Prellner, K.: Complement activation by pneumococci associated with acute otitis media.
Acta Pathol Microbiol Scand (C) 87, 213-216, 1979.
- VI. Johnson, U. & Prellner, K.: Purification of C-reactive protein on DEAE-cellulose by a simple two-step procedure utilizing the calcium-dependency of the protein.
Biochim Biophys Acta 495, 349-353, 1977.
- VII. Prellner, K.: C1q binding and complement activation by capsular and cell wall components of *S. pneumoniae* type XIX.
Acta Pathol Microbiol Scand (C). Accepted for publication.

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ABBREVIATIONS AND NOMENCLATURE

OME	otitis media with effusion
MEE	middle ear effusion

OME exists in an acute, a subacute and a chronic form and can further be characterized on the basis of the appearance of the MEE as purulent, serous or mucoid (Senturia et al., 1980). In the present investigation all acute OME were judged as purulent and will be referred to as purulent OME. All subacute and chronic OME exhibited serous to mucoid effusions and will be referred to as serous/mucoid OME.

CRP	C-reactive protein
C	complement
B	factor B
D	factor D
P	properdin
C $\bar{1}$ IA	C $\bar{1}$ inactivator
H	β 1H
I	C4b/C3b inactivator
C1q BA	C1q binding assay
C1q DT	C1q deviation test

The nomenclature used for complement factors is in agreement with accepted conventions (Austen et al., 1970; Alper et al., 1981). Fragments arising from C factors on activation are denoted by addition of a small letter to the symbol representing the C component in question, *e.g.* C3a, C3b, C4a and C4b. Enzymatic activity is designated with a bar over the number, *e.g.* C $\bar{1}$ s.

In certain experiments sera were chelated with ethylenediamine-tetra acetic acid (EDTA) to block C activation or with ethyleneglykolbis(aminoethylether)-tetra acetic acid (EGTA) to distinguish between classical and alternative pathway activation.

INTRODUCTION

EPIDEMIOLOGY AND MICROBIOLOGY OF OTITIS MEDIA

Otitis media with effusion (OME) is a common infectious disease in childhood, especially in children aged 1 to 2 years (Brownlee et al., 1969; Kaplan et al., 1973; Howie et al., 1975). In a prospective study of 2 565 children Teele and coworkers (1980) stated that during their first year almost half of the children had had at least one episode of OME. One third of the children had, at the age of 3, experienced three or more episodes. This is in agreement with other studies concerning Caucasians (Brownlee et al., 1969; Howie et al., 1976). Among Indian and Eskimo populations even higher incidences of OME are reported, while Negroes do not acquire OME as often as Caucasians (*cf.* Wiet et al., 1980).

Persistence of middle ear effusion (MEE) is frequent and one month after the OME diagnosis effusion is still present in 30-40% of the children (Pelton et al., 1977; Teele et al., 1980). In purulent OME *S. pneumoniae* (pneumococci) is the most frequent agent, accounting for 30-50% of the episodes, while *H. influenzae* is responsible for approximately 20% of OME in children from Sweden, Finland and the United States. Streptococci group A and *B. catarrhalis* are isolated in frequencies of up to 10% each (*cf.* Klein, 1980). *B. catarrhalis* has earlier been regarded as non-pathogenic. However, Coffey and coworkers (1966, 1967) and Kamme and coworkers (1971) showed *B. catarrhalis* to be etiologic in purulent OME in children and more frequently present in MEE than streptococci group A.

Relatively few types of pneumococci are responsible for most of the infections. The six most common types in children under the age of 3 are types 3, 6, 14, 18, 19 and 23 (Kamme et al., 1970; *cf.* Klein, 1980). OME

due to *H. influenzae* is usually (85%) caused by non-typable strains (Bjuggren & Tunevall, 1952; Grönroos et al., 1964; Kamme & Lundgren, 1971).

Relapsing purulent OME is usually due to pneumococci and the pneumococcal type is usually the same as in the primary episode. About 10% of children younger than 2 years with purulent OME due to pneumococci have relapsing otitis within one month after the onset of the primary episode. Purulent OME due to types 14 and 19 are more frequently followed by relapses than other types (Kamme et al., 1970).

Middle ear effusions from patients with serous/mucoid OME were earlier considered sterile, but with new techniques for culturing, pathogens have been isolated from approximately one fourth of these effusions (Liu et al., 1975; Healy & Teele, 1977; Riding et al., 1978).

PNEUMOCOCCAL ANTIGENS AND OPSONIC REQUIREMENTS

The pneumococcus is a gram-positive encapsulated bacterium. The capsule consists of polysaccharides. Eighty-three different capsular type antigens have been identified (*cf.* Henrichsen, 1979). Of the two major antigenic polysaccharides of the cell wall, peptidoglycan and teichoic acid (Baddiley, 1968), the latter is the part of the pneumococcal C-polysaccharide which forms complexes with CRP (Gotschlich & Liu, 1967; Brundish & Baddiley, 1968).

The virulence of pneumococci is largely dependent on its ability to resist phagocytosis in the host. For phagocytosis to occur the encapsulated pneumococcus must be opsonized by serum components, namely specific antibodies and certain C components. Thus in the presence of specific capsular antibodies the pneumococci are ingested by granulocytes and macrophages, but only slowly (Ward & Enders, 1933). In pneumococcal infections the C system is activated with formation of *inter alia* C3b, which is the most important opsonin for invasive pneumococci (Winkelstein et al., 1975). The rate of ingestion is markedly enhanced following the binding of C3b to the bacteria (Johnston et al., 1969; Shin et al., 1969).

Antibodies against specific capsular polysaccharides are often present in healthy children but the antibody levels are low as compared to those found in healthy adults (*cf.* Borgoño et al., 1978). In purulent OME in children increased levels of specific antibodies of the IgG, IgM and IgA classes are often found in serum (Sloyer et al., 1974; Sloyer et al., 1975; Branefors-Helander et al., 1975, 1980) and in the MEE (Sloyer et al., 1974; Sloyer et al., 1975).

CLASSICAL PATHWAY

ALTERNATIVE PATHWAY

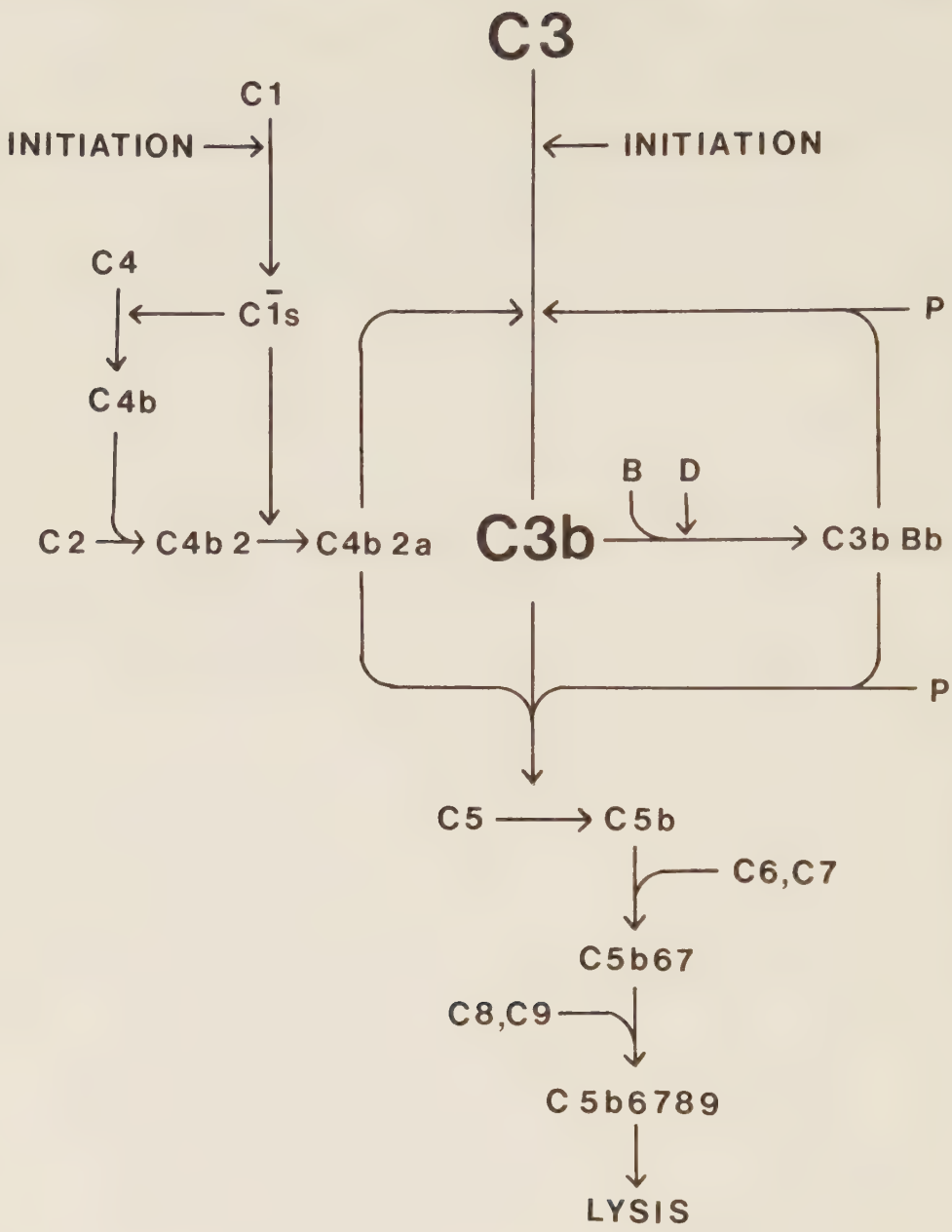


Fig. 1. Schematic presentation of the sequential activation of the classical and alternative pathways of the complement system.

THE COMPLEMENT SYSTEM

The C system is an essential part of the normal defence mechanisms in that it induces inflammatory response, enhances phagocytosis and causes lysis of certain bacteria. Biologically active components that can contribute to tissue damage are also formed on activation of the system.

The C system comprises a series of plasma proteins designated C1, C2, C3, C4, C5, C6, C7, C8, C9, B, D and P, some of which are proenzymes. Due to advances in protein chemistry most of the factors have been highly purified and physiochemically characterized.

The C factors are sequentially activated either through the classical or the alternative pathway. The first factors to be activated in the classical pathway are C1, C4 and C2, and in the alternative pathway, C3, B, D and P. The pathways converge at the C3 level and the reaction sequence C5-C9 is common to both pathways.

Activation is regulated by spontaneous decay of activated complexed components and by inhibitors. Hitherto, six control proteins have been identified: the C1 IA, I, H, the C4 binding protein, the anaphylatoxin inactivator and the s-protein.

Several C components, especially B, C1s and C3, react as acute phase proteins (Schutte et al., 1974). Thus, in some instances consumption of C factors may be masked by a concomitant acute phase reaction.

The classical pathway (*cf.* Fig. 1)(*cf.* Müller-Eberhard, 1975; Porter, 1977; Fearon & Austen, 1978). The first component of the classical pathway, C1, is a macromolecular complex of three proteins, C1q, C1r and C1s, held together with Ca^{2+} (Fig. 2)(Lepow et al., 1963; Naff et al., 1964). In serum an equilibrium exists

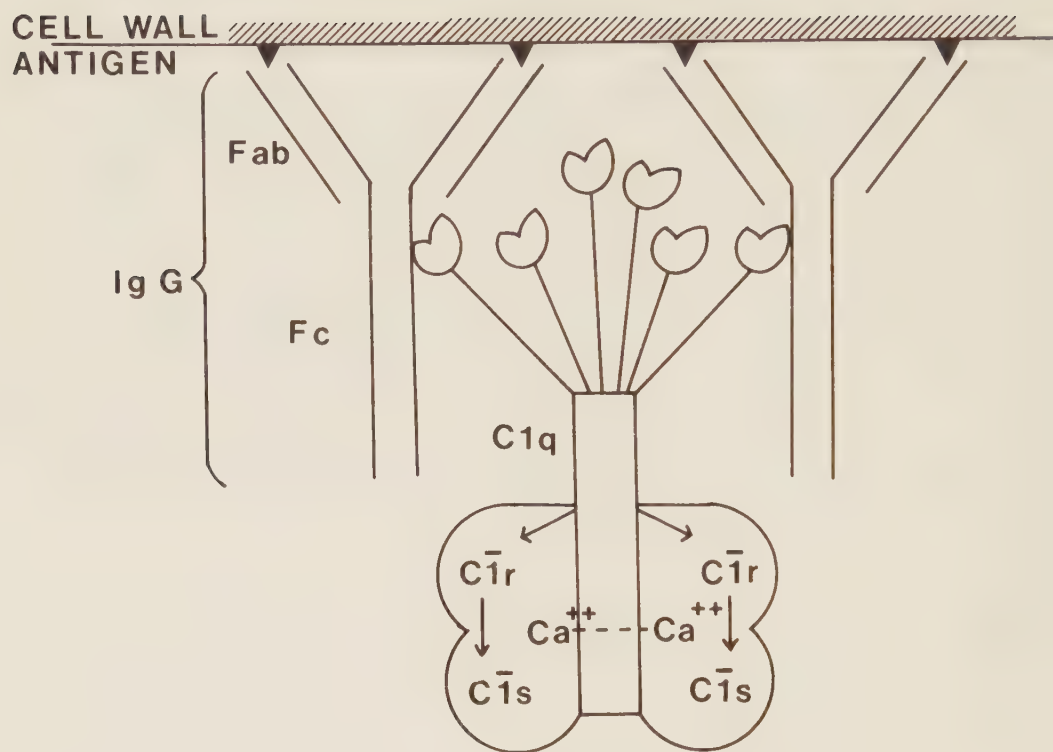


Fig. 2. Subcomponents of C1 and their reaction with cell surface associated IgG.

between free and macromolecularly bound C1 subcomponents. Current knowledge suggests this equilibrium to be strongly shifted towards the C1 macromolecule (Bartholomew & Esser, 1977).

The most important biological activators of C1 are immune complexes containing IgG or IgM (Augener et al., 1971). IgG and IgM have binding sites for C1q in their Fc regions (as illustrated for IgG in Fig. 2). One IgM molecule in complex with antigen is sufficient to activate C1, while at least two IgG molecules in close proximity are required (Borsos, 1971). The structure of C1q was revealed by chemical analysis and electron microscopy (Shelton et al., 1972; Yonemasu & Stroud, 1972): six peripheral globular structures, representing the Fc binding regions, are connected by fibrillary strands to a central portion (Fig. 2), representing the C1r and C1s binding sites of the molecule (Knobel et al., 1974; Reid & Porter, 1976).

C1r exists in native C1 as a proenzyme. On C1 activation C1r is converted to an enzyme, C1 $\bar{r}$, which acts on proenzyme C1s, then converted to C1 $\bar{s}$ (Sakai & Stroud, 1974; Valet & Cooper, 1974a,b). C1 $\bar{s}$ splits C4 into two fragments, C4a and C4b. The latter forms a complex with C2 which then becomes accessible for cleavage by C1 $\bar{s}$ leading to the formation of C4b2a (Gigli & Austen, 1969; Budzko & Müller-Eberhard, 1970; Patrick et al., 1970). C4b2a is the C3 convertase of the classical pathway (Polley & Müller-Eberhard, 1968) splitting C3 into C3a and C3b (Müller-Eberhard et al., 1967; Bokisch et al., 1969). The latter binds covalently to cell walls and immune complexes (Bokisch et al., 1975; Capel et al., 1978; Law et al., 1979).

Together with C4b2a, the C3b generates an enzyme which cleaves C5 into the fragments C5a and C5b (Cooper & Müller-Eberhard, 1970; Nilsson et al., 1975). C5b and C6 fuse to form a bimolecular complex (Lachmann &

Thompson, 1970; Podack et al., 1978a), which together with C7 gives rise to the trimolecular C5b67 complex. During the final steps C8 and C9 bind whereby the complex C5b6789 acquires membranolytic activity (Manni & Müller-Eberhard, 1969; Kolb & Müller-Eberhard, 1974; Podack et al., 1978b).

Regulator proteins can block the C activation at various levels. C1 IA inhibits C1r and C1s (Gigli et al., 1968; Harpel & Cooper, 1975). C4b and C3b are cleaved by I in cooperation with C4 binding protein and H as the respective cofactor (Cooper, 1975; Whaley & Ruddy, 1976; Weiler et al., 1976; Conrad et al., 1978; Fujita & Nussenzweig, 1979).

C1 is activated mainly by immune complexes but also by other entities such as proteolytic enzymes (Ratnoff & Naff, 1967; Laurell et al., 1978b), CRP in complex with pneumococcal C-polysaccharide or certain polycations (Kaplan & Volanakis, 1974; Siegel et al., 1975; Laurell et al., 1978b) and lipid A of gram-negative bacteria (Loos et al., 1974; Cooper & Morrison, 1978).

The alternative pathway (*cf.* Fig. 1) (*cf.* Götze & Müller-Eberhard, 1974; Fearon & Austen, 1978, 1980). This pathway was originally described by Pillemer and coworkers (1954, 1956) as the properdin system. It is activated by a variety of substances, such as microbial polysaccharides (Pillemer et al., 1955), lipopolysaccharides from gram-negative bacteria (Pillemer et al., 1955; Gewurz et al., 1968; Marcus et al., 1971) and teichoic acid from pneumococci (Winkelstein & Tomasz, 1978). This pathway is distinct from the classical pathway with which it converges at the C3 level. The C3 convertase of the alternative pathway is dependent on C3b or a C3-like protein and on B, D and Mg^{2+} (Müller-Eberhard & Götze, 1972). After interaction with C3b, B is cleaved by D and thus the C3 convertase C3bBb is formed (Vogt et al.,

1975; Vogt et al., 1977; Lesavre & Müller-Eberhard, 1978). This complex is labile (Fearon et al., 1973) but binding of P increases its stability (Fearon & Austen, 1975; Schreiber et al., 1975). C3bBb is the analogue of C4b2a of the classical pathway and converts native C3 to C3a and C3b. Cooperation of C3b and the C3bBb complex leads to cleavage of C5. Thus, the alternative pathway enters the C5-C9 sequence of the classical pathway (Daha et al., 1976; Medicus et al., 1976; von Zabern et al., 1979).

A controversial point is the formation of the initial C3 convertase. Several theories have been proposed (Fearon & Austen, 1975; Lachmann & Halbwachs, 1975; Pangburn & Müller-Eberhard, 1980; Janatova & Tack, 1981). Pangburn & Müller-Eberhard suggested that a C3-like protein plays a role in the formation of the initial C3 convertase.

An essential part of the alternative pathway is the C3b generating feedback mechanism (Fig. 1) called the amplification loop (Müller-Eberhard & Götze, 1972; Fearon et al., 1973; Nicol & Lachmann, 1973) in which C3bBb by cleaving C3 recruits more C3b for its own formation. This feedback mechanism can also be initiated by C3b obtained through activation of the classical pathway (Mak et al., 1977; Johnson, 1978).

Biological functions. The main functions of the C system are related to its ability to participate in the induction of an acute inflammatory response serving to limit the spread of injurious agents, including bacteria, and to facilitate the destruction of such agents by phagocytosis and/or lysis. These functions are clearly demonstrated by the striking susceptibility to severe bacterial infections of individuals with genetic C3 deficiency (Alper et al., 1970), or with hypercatabolism of this factor due to deficiency of I (Abramson et al., 1971).

On activation of C the biologically active components C3a and C5a which can sustain the inflammatory reaction, are formed (*cf.* Hugli & Müller-Eberhard, 1978). The C5a fragment is chemotactic for polymorphonuclear leukocytes (Chenoweth & Hugli, 1978). Polymorphonuclear leukocytes, macrophages and B-lymphocytes have receptors for C3b, and can thus bind particles coated with C3b (*cf.* Bianco & Nussenzweig, 1977). This immune adherence to phagocytes is the basis for C3b opsonization important in the host defence. Completion of C activation on the surface of certain cells results in lysis.

COMPLEMENT ACTIVATION IN DISEASE WITH SPECIAL REFERENCE TO PNEUMOCOCCAL INFECTIONS

Activation of the C system, as demonstrated with immunochemical and functional tests, has been described in a variety of conditions with an immunological background (*cf.* Schur, 1977; Whicher, 1978).

A prominent feature of immune complex diseases is the finding of decreased levels of classical pathway components in serum as a sign of activation by immune complexes. Although many bacterial infections are associated with normal or elevated serum levels of C, hypocomplementemia is seen in fulminant septic conditions. In gram-negative bacteremia mainly alternative pathway activation is observed attributable to effects of bacterial lipopolysaccharides (McCabe, 1973; Fearon et al., 1975). In septic shock, due to pneumococci, marked activation of both classical and alternative pathways has been reported. In adults with less severe pneumococcal infections, the C profile is consistent with activation of the alternative pathway while the early factors of the classical pathway are essentially normal (Hinz, 1956; Reed et al., 1976; Coonrod & Rylko-Bauer, 1977).

Pronounced C aberrations have been reported in children with purulent pneumococcal OME (Johnson et al., 1977). Children with relapsing purulent OME due to pneumococci had high concentrations of complexed $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ IA ($C\bar{1}r-C\bar{1}s-C\bar{1}$ IA). This complex is formed on activation of C1 (Laurell et al., 1978b) and is also present in small amounts in sera from healthy adults (Laurell et al., 1976) and children (II). Decreased levels of C1q with concomitantly normal or elevated levels of C1r and C1s were demonstrated in 60% of the children with relapsing purulent OME. Furthermore large amounts of complexes of proenzyme C1r-C1s were regularly found. The amounts of C1r-C1s complexes found in relapsing purulent pneumococcal OME

were generally larger than those observed in other diseases (Laurell et al., 1977).

The mechanisms responsible for the low C1q levels and the generation of C1r-C1s complexes have not been clarified nor have their significance in relapsing purulent OME.

AIMS OF THE INVESTIGATION

The object of the present investigation was to relate aberrations in serum complement of children with otitis media with effusion to interactions between bacterial substances and the complement system, thereby possibly elucidating some immunological mechanisms involved in relapsing purulent otitis media.

The investigation focused on the following questions:

1. Are the complement aberrations previously observed in children with purulent pneumococcal OME also present in
 - healthy children?
 - children with purulent OME due to other bacteria than pneumococci?
 - children with serous/mucoid OME?
 - adults with purulent pneumococcal OME or severe pneumococcal infections?
2. Are substances capable of interacting with complement present in serum and/or MEE from patients with OME?
3. Can bacteria encountered in purulent OME and/or specified pneumococcal constituents
 - bind C1q in the absence of antibodies?
 - activate complement through the classical and/or the alternative pathway?

MATERIALS AND METHODS

Several clinical entities (I, II, III) and, for reference purposes, a group of healthy children (II) were studied. Sera from 29 healthy children, from 16 children with purulent pneumococcal OME and from 11 children with purulent OME due to *H. influenzae* were investigated. Seventeen of the purulent OME episodes were relapses occurring within one month after a prior attack. Serum samples from 86 patients with serous/mucoid OME, which had lasted between 1 and 6 months, and 20 corresponding MEE were studied. We further investigated 16 adult patients with pneumococcal infections, 6 with purulent OME, 4 with pneumonia, 4 with meningitis and 2 with septic shock. Both acute and follow-up serum and plasma samples after 2-4 weeks were usually obtained.

Bacteria (IV, V) as pneumococci and *H. influenzae* were identified according to conventional methods. Pneumococcal types were determined by the Quellung reaction of Neufeld (1902). *H. influenzae* were typed by slide agglutination. *B. catarrhalis* were identified as described by Kamme (1970). The optical density of the bacterial suspensions used in the experiments was adjusted to $A_{(540)}=0.7$. The suspensions were centrifuged and resuspended in one tenth of the original volume. For pneumococci the bacterial concentration was approximately 3×10^9 per ml, as determined by a plating technique.

Pneumococcal cell wall constituents (VII) were prepared essentially as described by Winkelstein & Tomasz (1978). Teichoic acid was extracted from cell wall preparations with hot formamide (Fuller, 1938; Winkelstein & Tomasz, 1978).

Incubation of sera (IV, V, VII) with bacteria or bacterial constituents was carried out at 37°C for 30 or 60 minutes.

Serum, EDTA-plasma (EDTA 10 mmol/l)(I, II, III) and middle ear effusion samples (II) for analysis of C components and/or C1q binding substances were frozen in aliquots at -80°C within 6 hours of sampling.

Quantification of the complement components (I, II, III, V, VII), C1q, C1s, C4, C3, B and P, was done with electroimmunoassay (Laurell, 1972; Sjöholm, 1975; Laurell et al., 1978a). The values were given as per cent of the concentrations of a normal serum or plasma pool from healthy adults (Sjöholm, 1975; Laurell et al., 1978a).

C1 subcomponent complexes (I, II, III, V, VII), C1r-C1s and C1r-C1s-C1 IA, were demonstrated by crossed immunoelectrophoresis (Laurell et al., 1976) and quantificated with electroimmunoassay. Values were given in per cent of standard references (Laurell et al., 1979; Laurell et al., 1981).

C3 conversion (V, VII) was assessed by crossed immunoelectrophoresis (Ganrot, 1972) and evaluated by planimetry. The proportion of converted C3 was expressed as a percentage of the total area, for non-converted and converted C3, outlined by the precipitate. Values for C3 conversion were given after correction for the C3 conversion present in untreated paired controls.

Alternative pathway activation (V, VII) was studied in normal human serum to which Mg^{2+} EGTA (EGTA 10 mmol/l; MgCl_2 2.5 mmol/l) was added to prevent C1 activation (Fine et al., 1972), in C1q deficient serum (C1q level $< 3\%$) and in serum from a patient with genetic C2 deficiency.

C1q binding substances (I, II, IV, VII) were investigated with the C1q DT (Sobel et al., 1975) and with the C1q BA (Zubler et al., 1976). C1q binding equal to or exceeding that of aggregated IgG at a concentration of 100 $\mu\text{g/ml}$ was considered significant (II; Berglund

et al., 1980).

A method was designed to quantificate the uptake of C1q by intact bacteria (IV). A slightly modified procedure of that described by Sobel and coworkers (1975) for binding of radiolabelled C1q to sensitized sheep erythrocytes was used. The influence of temperature, incubation time and conductance on the binding of ^{125}I -labelled C1q to pneumococci and *E. coli* was investigated. With increasing temperature up to 37°C an increasing C1q uptake occurred. Ninety per cent of the total amount of C1q bound during 120 min incubation was taken up within the first 10 min. The highest level of C1q binding was obtained at conductance between 7 and 9 mS/cm. At conductance of 14 mS/cm the numerical values for C1q uptake were lower than at 7 mS/cm but the relation between the C1q uptake for different bacterial strains was not influenced (unpublished observation).

A temperature of 20°C , an incubation time of 20 min and a conductance of 7 mS/cm were used in the experiments.

The C1q binding capacity of bacterial cell constituents was investigated in a system with veronal buffered saline containing human serum albumin (VII). The investigation then proceeded as described by Sobel and coworkers (1975) for the C1q DT and by Zubler and coworkers (1976) for the C1q BA.

CRP (I, III, VI) and α_1 -antichymotrypsin (I) were quantificated with electroimmunoassay (Laurell, 1972) whereby values below 12 mg/l and 0.6 g/l, respectively, were considered normal.

COMPLEMENT COMPONENTS AND C1q BINDING SUBSTANCES IN PATIENTS (I, II, III)

Complement components in serum

C1r-C1s-C1 IA complexes are present in normal sera (Laurell et al., 1977). C1r-C1s complexes are only found in 1% of normal adult sera (Sjöholm, 1979). In both adults and children the levels of C1q, C1s, C4, C3, B and P were given in per cent of their concentrations in serum or plasma pools from healthy adults (Sjöholm, 1975; Laurell et al., 1978a).

In healthy children (II), the levels of C1s, C4, C3 and P and the amounts of C1r-C1s-C1 IA complexes were close to normal levels in adults. The mean level of C1q was $86 \pm 15\%$ (mean $\pm$ S.D.) of that in adults. C1r-C1s complexes were observed in 3 of the 29 children but only in low amounts.

There were no significant differences in C components between children with purulent OME due to pneumococci and *H. influenzae* (I).

In non-relapsing purulent OME in children the mean serum levels of C1q, C1s, C4, C3 and P in the acute phase did not differ significantly from values obtained in healthy children. However, a marked disproportion between C1s and C1q was registered in all sera but one. The amounts of C1r-C1s-C1 IA complexes were increased in 5 and C1r-C1s complexes were present in 7 of 10 patients.

In relapsing purulent OME in children the levels of C1q in acute serum samples were lower than in healthy children with mean values of 69 and 67% for pneumococci and *H. influenzae*, respectively. In all but one serum C1q was disproportionally low compared to C1s, and the C1s-C1q discrepancy significantly larger than in healthy children. Furthermore, P levels were

significantly lower in these patients than in healthy children while C3 and B were increased in many of the children with relapsing purulent OME.

Seven of 17 patients demonstrated C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes in quantities exceeding the upper normal limit. C $\bar{1}$ r-C $\bar{1}$ s complexes were found in 15 of 17 patients and frequently in amounts clearly exceeding those demonstrated in the non-relapsing purulent OME.

In follow-up samples (2-3 weeks) the C factor levels tended to normalize. However, in relapsed cases C $\bar{1}$ q levels were still low, with a mean of 74% for pneumococcal and 82% for *H. influenzae* purulent OME. The amounts of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA and C $\bar{1}$ r-C $\bar{1}$ s complexes had most frequently decreased, but the opposite was also observed.

In children with serous/mucoid OME (II), as in healthy children, the mean C $\bar{1}$ q level was approximately 85% of the adult reference. However, in serous/mucoid OME there was a disproportion between C $\bar{1}$ s and C $\bar{1}$ q levels, which differed significantly from that seen in healthy children. C4 and C3 were normal, while P was significantly lower than in healthy children. In 20 of 86 children C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes were present in increased amounts and 35 of the 86 sera contained C $\bar{1}$ r-C $\bar{1}$ s complexes, but in lower amounts than in relapsing purulent OME as judged by crossed immunoelectrophoresis.

In follow-up samples (4 weeks) increased levels of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA and C $\bar{1}$ r-C $\bar{1}$ s complexes were less frequently demonstrable. The decrease in the C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes was most pronounced in patients without MEE at follow-up.

In adults with purulent OME due to pneumococci (III) the levels of C $\bar{1}$ q, C $\bar{1}$ s, C4, C3, B and P were essentially within normal ranges, as were the levels of

C1r-C1s-C1 IA complexes.

Of adults with more severe pneumococcal infections (III) patients with pneumonia or meningitis exhibited essentially normal concentrations of C1q, C4 and C3. However, in the acute phase, C1s and B concentrations were often increased while P was decreased. Large amounts of C1r-C1s-C1 IA complexes were found in all but one of the patients, and low amounts of C1r-C1s complexes were demonstrated in one patient.

Marked hypocomplementemia was found in connection with shock syndrome developing in 2 patients. Large amounts of C1r-C1s-C1 IA complexes were present in one patient but not in the other. No C1r-C1s complexes were present in the acute phase.

On healing, the C factor levels normalized in all patients.

C1q binding substances in serum and middle ear effusion (I, II)

C1q binding substances were measured by C1q DT and by C1q BA. Discrepancies in results between the two assays were recorded in some patients. C1q BA mainly detects immune complexes, while C1q DT is known to reveal not only the presence of immune complexes but also that of other C1q binding substances such as bacterial constituents (Sobel et al., 1975; Zubler et al., 1976).

With the C1q DT, significant amounts of C1q binding substances in serum were demonstrated in 1 of 29 healthy children. In purulent OME due to pneumococci and *H. influenzae* C1q binding substances were demonstrable ($> 100 \mu\text{g/ml}$) in 9 of 16 and in 2 of 9 acute sera, respectively. The levels were significantly higher in patients with purulent pneumococcal OME than in patients with purulent OME due to *H. influenzae*. In

children with serous/mucoid OME C1q binding substances were demonstrated in 16 of 57 sera.

In follow-up samples (2-4 weeks) the C1q binding capacity demonstrated by C1q DT had decreased in 10 and increased in 6 of 16 patients with purulent OME while in patients with serous/mucoid OME a decrease was recorded in 7 and an increase in 11 of 31 patients.

Sera containing C1q binding substances of more than 100 µg/ml showed significantly higher levels of C1r-C1s complexes than sera containing less C1q binding substances. However, C1r-C1s complexes were demonstrated also in sera where C1q DT was negative. In some sera containing more than 100 µg/ml of C1q binding substances no C1r-C1s complexes could be demonstrated.

With the C1q BA, quantities exceeding 100 µg/ml were found in 1 of 29 healthy children, in 3 of 27 children with purulent and in 5 of 57 children with serous/mucoid OME (unpublished results).

In serous MEE (II), C1q binding substances were detected by C1q DT and/or C1q BA in 17 of 20 effusions. The values ranged from 130 to > 2000 µg/ml. C1q binding substances were demonstrated in the corresponding serum of 6 of these 17 patients. In the remaining 3 patients C1q binding substances could not be detected in either serum or effusion.

CRP (I, III, IV) and α_1 -antichymotrypsin (I)

No increase of CRP was observed in children with serous/mucoid OME (unpublished results), or in patients with purulent OME due to *H. influenzae* (I). CRP levels were moderately increased in 10 of 16 children and in 3 of 6 adults with purulent pneumococcal OME (I, III). All adults with more severe pneumococcal infections demonstrated high CRP levels (III).

α_1 -antichymotrypsin (I), only investigated in purulent OME, was above normal levels in 14 of 16 patients with purulent pneumococcal OME and in 5 of 11 with purulent OME due to *H. influenzae*.

DISCUSSION

Although several studies on C in umbilical cord serum have been published, information on C component levels in infancy and childhood is scarce. In cord serum moderately low concentrations of C components have been reported (Fishel & Pearlman, 1961; Adinolfi, 1972; Ballow et al., 1974; Yonemasu et al., 1978; Davis et al., 1979). Some investigators recorded adult levels of C1q, C1 and C4 within 4 days of birth (Ballow et al., 1974; Yonemasu et al., 1978). In contrast C3 remained below adult levels until 1 month after birth from whence it increased to adult or even higher levels (Yonemasu et al., 1978). In another study C1q did not reach adult levels until the age of 3 years and C3 and P were still low at the age of 6 months (Davis et al., 1979). In our study C1q was moderately low in healthy children between 2 and 10 years of age while levels of C1s, C3, C4 and P (II) were close to those reported in adults (Sjöholm, 1975; Laurell et al., 1978a).

Considering these somewhat contradictory results, C component levels in the present investigation were expressed in per cent of the concentration in a reference pool of sera from healthy adults (Sjöholm, 1975; Laurell et al., 1978a).

The serum concentrations of C1r-C1s-C1 IA complexes in healthy children (II) were the same as in adults (Laurell et al., 1979). Low amounts of C1r-C1s complexes were found in approximately 10% of sera from healthy children (II) as compared to 1% in adults (Sjöholm, 1979).

Children with purulent pneumococcal OME exhibited low C1q compared to C1s, complexes of C1r-C1s and increased levels of C1r-C1s-C1 IA complexes (Johnson et al., 1977; I). The present study showed that this pattern is not restricted to purulent OME caused by pneumococci but is also seen with *H. influenzae* and in children with serous/mucoid OME (I, II). However, in purulent non-relapsing and in serous/mucoid OME C aberrations were less frequent and less pronounced than in purulent relapsing OME (I, II). In the latter and in serous/mucoid OME, P was lower than in children with a single attack of purulent OME.

In sera from patients with relapsing and non-relapsing purulent OME as well as in sera from serous/mucoid OME (I, II), the abnormal C profile may to some extent be explained by the presence of C1q binding substances. Such substances were more frequently found in purulent pneumococcal OME, both non-relapsing and relapsing, than in the other clinical groups (I, II). The presence of these substances in relapsing purulent OME is in agreement with the detection of C1q binding substances in serum from children with other relapsing infections (Delire & Masson, 1977).

The presence in serum of C1q binding substances in purulent as well as in serous/mucoid OME in children and the fact that the C disorders were similar in these clinical entities may indicate that the mechanisms are similar in the various forms of otitis media.

Veltri & Sprinkle (1976) suggested that serous OME is mediated by immune complexes. In chinchillas, immune complexes injected into the middle ear caused acute local inflammatory changes (Mravec et al., 1978). The necessary constituents for the formation of immune complexes, namely bacterial antigens (Liu et al., 1975; Healy & Teele, 1977; Riding et al., 1978) and immunoglobulins, have been found in serous MEE (Veltri &

Sprinkle, 1973, 1976). Maxim and coworkers (1977) gave evidence for the presence in serous MEE of immune complexes containing C3b. The large amounts of C1q binding substances demonstrated in 85% of serous MEE (II) supports this concept. Thus, it seems likely that immune complexes in the middle ear contribute to the perpetuation of serous OME.

The abnormal C profile in serum of children with purulent pneumococcal OME (Johnson et al., 1977; I) could not be demonstrated in adults with this disease (III). In adults with more severe pneumococcal infections evidence of C1 activation was found, but the levels of C1q were largely normal (III).

The high levels of C1r-C1s complexes in children as compared to the absence of such complexes in virtually all adults with pneumococcal infections suggest that different mechanisms may operate at the C1 level in the two age groups.

EXPERIMENTS ON COMPLEMENT ACTIVATION AND C1q BINDING BY BACTERIAL CONSTITUENTS (IV, V, VI, VII)

Activation of C1

Intact pneumococcal cells (V), types 1, 3, 6, 14, 18, 19 and 23, were incubated with normal human serum. Decreased C4 values and formation of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes (V), signifying classical pathway activation (Laurell et al., 1978b), were demonstrated. The values for C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA ranged from 30% (type 1) to 45% (type 19) of the reference (Laurell et al., 1979).

Pneumococcal cell constituents (VII) such as specific capsular polysaccharide and preparations of pneumococcal cell wall, teichoic acid and residual cell wall all induced C1 activation in normal serum. None of these preparations activated C1 in the absence of antibodies, as demonstrated in experiments with hypogammaglobulinemic serum.

CRP-teichoic acid complexes (VI, VII) were obtained by incubation of the teichoic acid preparation with highly purified CRP. The latter was prepared by a simple two-step procedure on DEAE cellulose columns which utilizes the difference in binding of CRP to DEAE in the presence and absence of Ca²⁺ (VI). The complex formation of CRP with teichoic acid was demonstrated by crossed immunoelectrophoresis using anti-CRP in the gel.

Addition of CRP-teichoic acid complexes to hypogammaglobulinemic serum resulted in an increase of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes in the serum, indicating activation of C1 by the CRP-teichoic acid complex.

Activation of C3

C3 conversion was studied in sera with an intact classical pathway and in sera where the pathway was in-

operative through deficiency of early factors or blocked by chelation with Mg^{2+} EGTA.

Intact pneumococcal cells (V) of all types studied, induced C3 conversion to approximately the same extent in sera in which the classical pathway was inoperative. Heat killed and viable pneumococci were equally potent in activating the C system.

All pneumococcal cell constituents (VII) except specific capsular polysaccharide converted C3 in sera in which the classical pathway was inoperative.

C3 conversion was more pronounced in serum with an intact classical pathway and then also occurred on incubation with specific capsular polysaccharide. In all instances where C3 conversion was registered a concurrent decrease in P was seen (V, VII).

C1q binding capacity

Eighty-seven different bacterial strains were investigated (IV; cf. Fig. 3). For reference purposes some species not commonly associated with purulent OME were included.

Non-typable *H. influenzae* showed the highest C1q binding capacity with values of around 90%. *H. influenzae* types a-f demonstrated less C1q binding and greater variation between strains. All strains of *B. catarrhalis* exhibited high and constant C1q binding values. The C1q uptake by different pneumococcal types varied. All type 3 strains gave constantly low values while marked variation was noted in types 6, 14, 19 and 23. A total of twenty strains of types 6, 19 and 23 were tested and eleven of these demonstrated high C1q binding capacity (Fig. 3).

Strains of *E. coli*, *S. aureus*, *S. saprophyticus* and *S. epidermidis*, streptococcus group A, B, C and G and

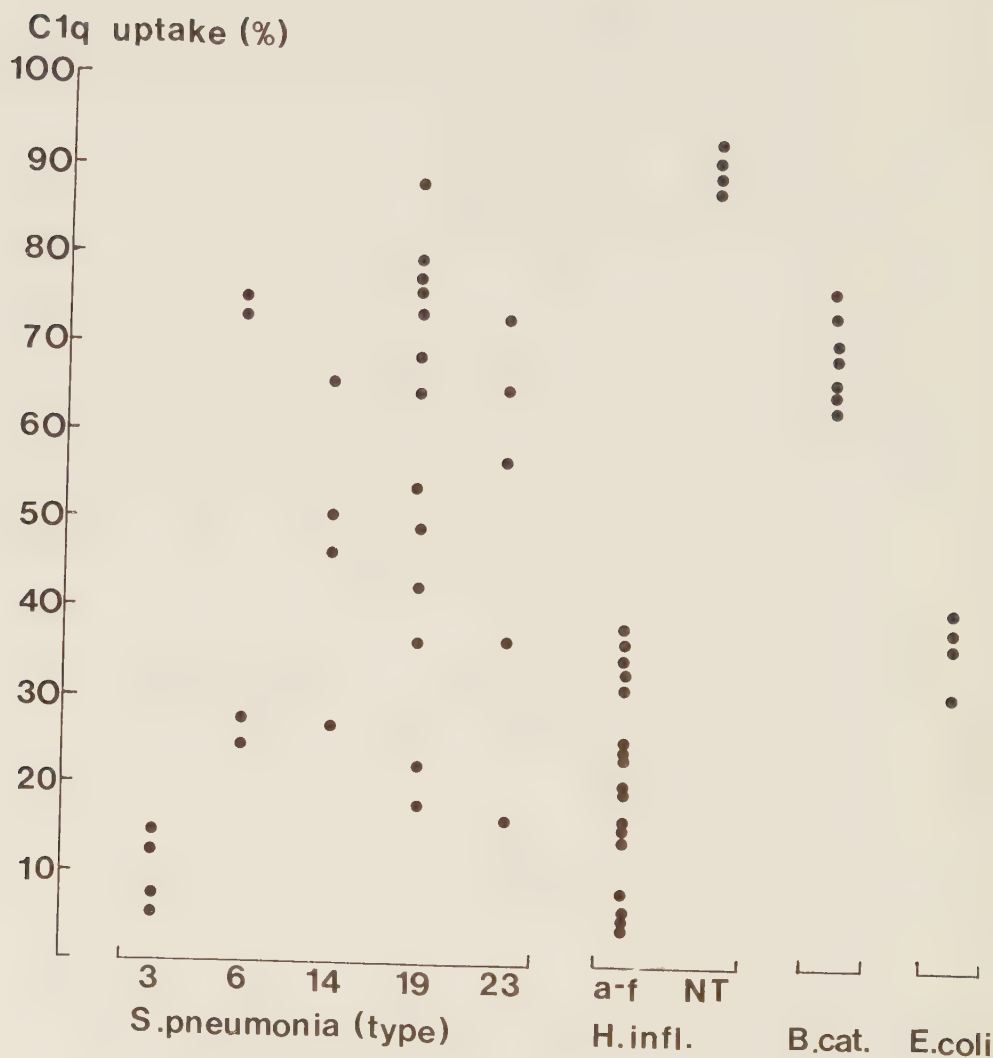


Fig. 3. C1q binding capacity of various bacteria often encountered in purulent OME in children (*S. pneumoniae*; non-typable, NT, *H. influenzae*; *B. catarrhalis*). For reference purposes *H. influenzae* types a-f and *E. coli* were included.

S. faecalis bound C1q to a much lower extent than non-typable *H. influenzae*, *B. catarrhalis* and most of the pneumococci tested.

Preparations of type specific capsular polysaccharide, pneumococcal cell wall, teichoic acid and residual cell wall from pneumococcus type 19 were analyzed for C1q binding (VII). In the C1q DT all cell wall preparations showed C1q binding capacity, while the type specific capsular polysaccharide did not. Teichoic acid was the most potent C1q binding pneumococcal substance. No C1q binding capacity was found when these preparations were analyzed with the C1q BA.

DISCUSSION

Classical pathway activation

The increased amounts of C1r-C1s-C1 IA complexes recorded on incubation of normal serum with the pneumococcal types 1, 3, 6, 14, 18, 19 and 23 (V) signify that classical pathway activation occurred (Laurell et al., 1978b). This was further supported by the decrease in C4 (V). Stephens and coworkers (1977) also found utilization of C4 by pneumococci types 3, 6, 18, 19 and 23, but not by types 1 and 14. In the assay systems used, variations in specific capsular antibody levels may provide sufficient explanation for the differences found. That heat killed as well as viable pneumococci activated C (V) indicates that activation was not induced by bacterial enzymes.

Sera with intact classical pathway exhibit larger opsonic activity against pneumococci than sera with the classical pathway blocked (Giebink et al., 1977). This is supported by the present finding of a more pronounced C3 conversion by pneumococci in normal serum than in C2 deficient serum or in normal serum with C1 activation blocked by Mg^{2+} EGTA (V).

It was suggested in early studies that type specific pneumococcal antibodies do not bind complement (Horsfall & Goodner, 1936; Stats & Bullowa, 1942). However, the present investigation clearly demonstrates activation of C1 on incubation of normal human serum, but not of hypogammaglobulinemic serum, with type specific capsular polysaccharide from pneumococci type 19 (VII).

Activation of C1 in normal human serum, but not in hypogammaglobulinemic serum, on incubation with pneumococcal teichoic acid or residual cell wall preparations can possibly be ascribed to the formation of antigen-antibody complexes, since cross reacting antibodies to these substances are common in normal serum (Yount et al., 1968; Decker et al., 1972).

Although teichoic acid and residual cell wall preparations did not activate C1 in the absence of antibodies, they were able to bind purified C1q (VII). These findings strongly suggest that teichoic acid is capable of binding C1q without activating C1.

Addition of CRP to the teichoic acid preparation induced C1 activation also in hypogammaglobulinemic serum (VII). Teichoic acid is known to be the part of the pneumococcal C-polysaccharide which interact with CRP (Gotschlich & Liu, 1967; Brundish & Baddiley, 1968) and earlier studies have shown that CRP-C-polysaccharide complexes are capable of activating the classical pathway (Kaplan & Volanakis, 1974; Osmand et al., 1975).

Alternative pathway activation

Pneumococcal types have been reported to vary in their capacity to activate the alternative pathway (Fine, 1975; Stephens et al., 1977). In the present study no such variation was found for the pneumococcal types 1, 3, 6, 14, 18, 19 and 23. In the present

study (V) type 1 pneumococci activated the alternative pathway, which is in agreement with the results of several investigators (Edwards & Stark, 1977; Stephens et al., 1977) but contrary to the finding of Fine (1975). Such discrepancies in results with individual pneumococcal types may be due to differences in the investigated strains or in the test sera used.

Incubation of serum with pneumococci resulted in a decrease in P indicating that P was consumed through alternative pathway activation. The P consumption was more pronounced in serum with an intact classical pathway than in serum in which the activation of C3 was mediated only through the alternative pathway (V). This might be explained by the contribution of additional C3b, generated through the classical pathway, triggering the C3b feedback mechanism (Fig. 1)(Mak et al., 1977; Johnson, 1978).

The pneumococcal cell constituents responsible for alternative pathway activation are not well defined. Winkelstein and coworkers (1976) found that purified soluble capsular polysaccharide types 1, 4 and 25 activated the alternative pathway whereas types 2, 3, 14 and 19 did not. In the present study capsular polysaccharide type 19 did not activate the alternative pathway (VII).

It has been proposed that cell wall peptidoglycan, rather than teichoic acid, as investigated in group A streptococci and staphylococci, accounts for the alternative pathway activation by gram-positive cocci (Greenblatt et al., 1978; Verbrugh et al., 1979; Verbrugh et al., 1980). On the other hand, in pneumococci, Winkelstein & Tomasz (1978) found teichoic acid and not peptidoglycan to be the more effective activator of the alternative pathway in guinea pig serum. We found teichoic acid preparation to be a potent activator of the alternative pathway in human serum (VII) which would support the findings of

C1q binding capacity

Some bacteria, including *E. coli*, are reported to interact directly with C1 (Bredt et al., 1977; Loos et al., 1978). Lipopolysaccharide preparations from this bacterium bound C1q resulting in activation of C1 (Müller-Eberhard et al., 1971; Loos et al., 1972; Loos et al., 1974).

Bacteria closely associated with purulent OME were capable of binding C1q in the absence of antibodies (IV). Teichoic acid from pneumococci demonstrated more pronounced C1q binding than the other polysaccharides (VII), and could thus be responsible for the main binding of C1q in pneumococci.

In the present study the C1q binding capacity of bacteria usually encountered in purulent OME was more pronounced than that of the *E. coli* strains tested. A striking difference between typable and non-typable *H. influenzae* was observed in that the latter exhibited a far more pronounced C1q binding capacity than the former (IV; Fig. 3). Noteworthy, 85% of purulent OME due to *H. influenzae* is caused by non-typable strains (Bjuggren & Tunevall, 1952; Grönroos et al., 1964; Kamme & Lundgren, 1971).

GENERAL DISCUSSION

The various forms of OME in children were associated with complement activation in serum (Johnson et al., 1977; I, II). Thus the activation of the classical pathway was demonstrated by increased amounts of $C\bar{1}r-C\bar{1}s-C\bar{1}$ IA complexes (Laurell et al., 1979) while the moderately decreased P indicated additional involvement of the alternative pathway. In severe pneumococcal infections in adults a more extensive formation of $C\bar{1}r-C\bar{1}s-C\bar{1}$ IA complexes was seen than in purulent pneumococcal OME in children (III).

The present investigation focused on possible reaction mechanisms in pneumococcal infections, since the pneumococcus is the most important pathogen in purulent OME and because it has been more thoroughly studied in relation to the C system than have other bacterial species associated with purulent OME.

Normal human serum, both in adults and children, usually contain type specific antibodies to pneumococci (Borgoño et al., 1978). Antibodies to bacterial cell wall polysaccharides, like teichoic acid and peptidoglycan, are also commonly demonstrated in sera from healthy adults (Yount et al., 1968; Decker et al., 1972). Thus, the classical pathway activation in purulent pneumococcal OME in children and in more severe pneumococcal infections in adults could be due to antigen-antibody reactions. *In vitro* incubation of normal human serum with pneumococci and with pneumococcal antigen preparations resulted in C1 activation (V, VII). Complex formation between CRP and pneumococcal C-polysaccharide might also account for classical pathway activation (Kaplan & Volanakis, 1974; VII). Direct activation of C1 by pneumococcal constituents without participation of antibodies or CRP, could not be demonstrated (VII).

The moderately low P found in children with relapsing purulent and serous/mucoid OME (I, II) and in adults with more severe pneumococcal infections (III) indicated involvement of the alternative pathway. This could possibly be due to the C3b feedback mechanism initiated by the C3 convertase of the classical pathway (Fig. 1)(Mak et al., 1977; Johnson, 1978). On the other hand, *in vitro* activation of the alternative pathway is elicited by pneumococci of types commonly associated with purulent OME in children (Fine, 1975; Edwards & Stark, 1977; Stephens et al., 1977; V) as well as by pneumococcal cell wall constituents (Winkelstein & Tomasz, 1978; VII).

In children with purulent relapsing OME the most striking feature of the C profile was the finding of low C1q levels and high levels of C1r-C1s complexes (Johnson et al., 1977; I). In adults with purulent pneumococcal OME this C profile was not seen, in fact no C aberrations were registered (III). Noteworthy, purulent OME in adults is virtually never relapsing.

The low C1q levels demonstrated in sera from children with purulent relapsing OME indicate that the C1q synthesis was decreased and/or that the C1q was consumed. C1q levels tended to increase in the follow-up samples but did not reach the levels found in healthy children (I, II). Although the possibility of a reduced C1q synthesis cannot be ruled out, the finding of substances capable of binding C1q, in MEE and sera (I, II), favours the view that C1q is consumed in the disease process. This is supported by the finding that intact bacteria associated with purulent OME, as well as pneumococcal cell constituents, were found to bind C1q *in vitro* (IV, VII).

The C1q binding capacity of serum from patients with purulent or serous/mucoid OME (I, II) might be due to the presence of soluble bacterial constituents, circulating immune complexes and/or of other factors. The

C1q binding substances could generally be demonstrated with C1q DT while the C1q BA was usually negative (I, II). The higher specificity of C1q BA as compared to C1q DT for the detection of immune complexes (Sobel et al., 1975; Zubler et al., 1976) favours the possibility that other C1q binding substances were present in the circulation of the patients. The possibility that the C1q binding capacity as demonstrated by C1q DT in serum from children with purulent or serous/mucoid OME was partly due to the presence of C1r-C1s complexes could not be excluded. It has been shown that native C1r-C1s complexes in certain pathological sera can combine with added C1q (Laurell et al., 1976). However, C1q binding substances were present in sera from children also when no C1r-C1s complexes were found (I).

C1r-C1s complexes appear when C1q is low as compared to C1r and C1s and have earlier been demonstrated in hypogammaglobulinemia, chronic urticaria and in selective C1q deficiency (Laurell et al., 1976; Loos et al., 1980). Increased production of C1r and C1s in relation to C1q would result in formation of C1r-C1s complexes. The increased levels of CRP and α_1 -anti-chymotrypsin in many of the children with purulent pneumococcal OME (I) showed that an acute phase reaction was present. It is known that the production of both C1s and C1q increases in the acute phase of inflammation, but C1s levels rise relatively more than C1q levels (Schutte et al., 1974). Such a mechanism may contribute to the appearance of C1r-C1s complexes, but cannot explain the low C1q values in relapsing purulent OME.

A recent study (McKay et al., 1981) describes the generation of C1r-C1s complexes from normal human serum on a heparin-Sepharose^R chromatography column, where C1q remained bound to heparin. In normal human serum the C1qrs complex is in a dissociation equilibrium with free C1 subcomponents (Bartholomew & Esser, 1977). Thus, in purulent OME, one could postulate a

competition between bacterial cell constituents and C1r-C1s for the available C1q. If C1q bound to bacterial substances is prevented from association with C1r and C1s, C1r-C1s complexes could be formed. The binding of C1q by pneumococcal teichoic acid might initiate such a course of events.

The encapsulated pneumococcus must be opsonized by serum components for phagocytosis to occur (Ward & Enders, 1933). C3b is the most important opsonin for invasive pneumococci (Johnston et al., 1969; Shin et al., 1969; Winkelstein et al., 1975) and is generated through activation of the classical and/or the alternative pathway.

There are several possibilities for the formation of C3b in pneumococcal OME. Intact pneumococci and pneumococcal cell constituents can activate the alternative pathway (Fine, 1975; Edwards & Stark, 1977; Stephens et al., 1977; Winkelstein & Tomasz, 1978; V, VII)(Fig. 4). Furthermore, these bacterial antigens can together with specific antibodies in MEE as well as in serum (Sloyer et al., 1974; Branevors-Helander et al., 1975) form immune complexes, which activate the classical pathway. Complexes between CRP and pneumococcal C-polysaccharide constitute a third possible C activator (Kaplan & Volanakis, 1974; VII) (Fig. 4).

The finding of low C1q with simultaneously high levels of C1r-C1s complexes in children with purulent relapsing OME might imply a disturbance of the classical pathway activation resulting in reduced opsonization of the pneumococci. Substances capable of binding C1q were present in MEE and serum of children with OME. The nature of these substances is yet not clear. However, pneumococcal cell wall constituents were able to bind C1q *in vitro* and did not activate C1 in the absence of antibodies (VII). The C profile in OME could be explained by the presence of both C

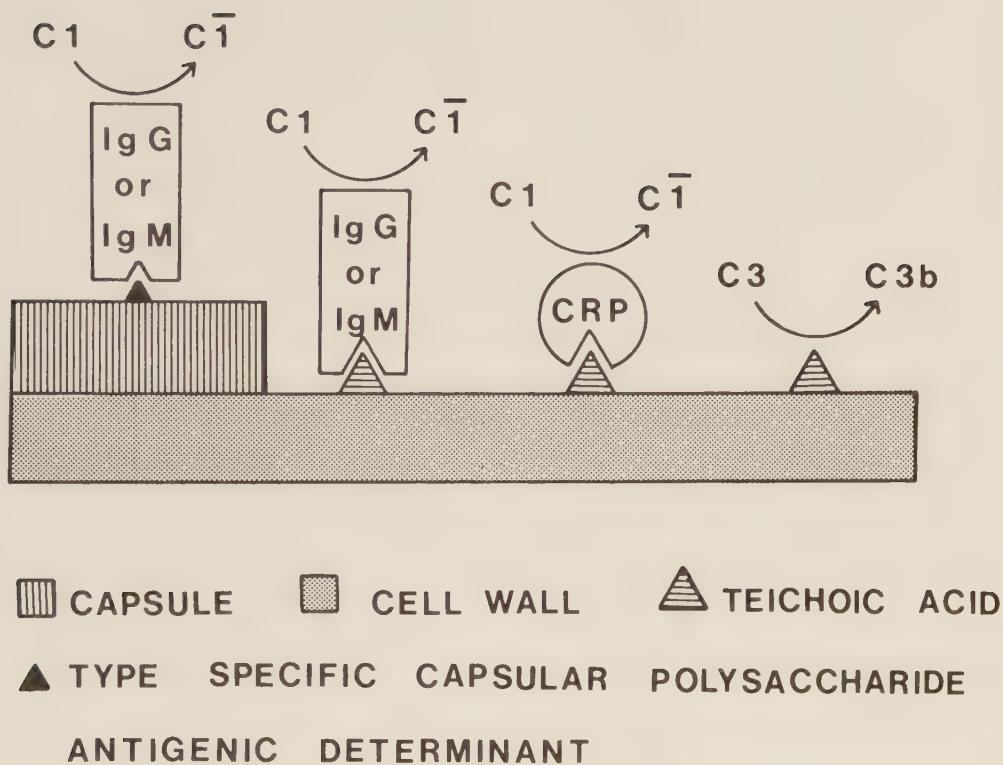


Fig. 4. Schematic presentation of the complement activation by pneumococcal antigens.

activating substances, such as immune complexes, and of substances with C1q binding but not C activating properties, in serum and/or MEE. If antigen-antibody complexes are in relative excess C activation will occur. C1r-C1s complexes are formed if C1q binding substances predominate over C activating substances. The frequent finding of C activation and infrequent demonstration of C1r-C1s complexes in adults with pneumococcal infections (III) would support this hypothesis.

Serum antibodies were not studied in the adult patients. However, most normal sera from healthy adults contain specific pneumococcal antibodies in quantities far exceeding those in children (Borgoño et al., 1978). Interestingly, pneumococcus type 3, not usually encountered in purulent relapsing OME but often as causative agent in primary purulent OME (Kamme et al., 1970), is not only highly immunogenic (Borgoño et al., 1978) but also exhibits low C1q binding capacity (IV).

Although the C aberrations tended to normalize, low C1q levels and C1r-C1s complexes persisted in many patients 2-4 weeks after the current purulent OME episode (I). One may assume that, regardless of the reason for the low C1q levels, bacteria invading the middle ear of a child with low C1q, are less easily phagocytized due to the impaired activation of the classical pathway, and thus more prone to cause infection than in a child with normal C1q level.

GENERAL SUMMARY

Otitis media with effusion (OME) is a common disease in childhood. *S. pneumoniae* is the most common agent of purulent OME and approximately 10% of children younger than 2 years with purulent OME due to pneumococci have relapsing OME. For phagocytosis to occur the encapsulated pneumococcus must be opsonized by specific antibodies and C3b. The latter is formed on activation of the complement system either through the classical or the alternative pathway. Pronounced complement aberrations have been reported in children with purulent pneumococcal OME.

The aim of this study was to investigate the occurrence of serum complement aberrations and C1q binding substances in children with purulent and serous/mucoid OME. For reference purposes, healthy children and adults with pneumococcal infections were studied. Furthermore, interactions between bacterial constituents and the complement system were studied *in vitro* in order to elucidate possible mechanisms for the complement aberrations found in OME.

Children with various forms of OME exhibited regularly low C1q compared to C1s, and frequently abnormal complexes of C1r-C1s, increased levels of C1r-C1s-C1 IA complexes, decreased properdin levels and in 30-50% of the sera presence of C1q binding substances. The complement aberrations in children with OME were most pronounced in relapsing purulent OME and were virtually never seen in adults with pneumococcal OME or other pneumococcal infections.

The increase of C1r-C1s-C1 IA complexes indicates activation of the classical pathway, while the reduced properdin signifies alternative pathway activation. However, the finding of reduced C1q and simultaneously large amounts of C1r-C1s complexes implies a disturbance of the classical pathway activation.

In vitro activation of the classical pathway occurred in normal serum with all tested pneumococci and cell constituents of pneumococcus type 19. In hypogammaglobulinemic serum classical pathway activation was induced by complexes between teichoic acid from pneumococcal cell walls and C-reactive protein. Activation of the alternative pathway was obtained with all types of pneumococci tested and with different cell wall constituents of pneumococcus type 19, but not with specific capsular substance type 19.

Intact pneumococci types 6, 14, 19 and 23 were shown to effectively bind C1q in the absence of antibodies as were other bacteria encountered in OME, such as non-typable *H. influenzae* and *B. catarrhalis*. *H. influenzae* types a-f and *E. coli* demonstrated less C1q binding capacity.

Cell wall constituents of pneumococcus type 19 effectively bound C1q without participation of antibodies but did not induce activation of C1 when incubated in hypogammaglobulinemic serum.

Thus, in pneumococcal OME there are several possibilities for the formation of C3b through classical and alternative pathway activation. However, pneumococcal cell wall constituents can bind C1q without activation of the complement system which could explain the low C1q levels in relapsing pneumococcal OME. This would result in reduced opsonization and thus phagocytosis of the infecting bacteria.

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COMPLEMENT ABERRATIONS IN SERUM FROM CHILDREN WITH OTITIS DUE
TO S.PNEUMONIAE OR H.INFLUENZAE

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ABSTRACT

Complement components, C1q, C1s, C3, C4, factor B and properdin were measured together with C1 subcomponent complexes and C1q binding substances in acute and convalescent samples from patients with relapsing and non relapsing otitis media due to S.pneumoniae and H.influenzae. Analysis of C1 subcomponent complexes together with the finding of low C1q levels gave evidence for a disturbed C1 function in acute OME. Furthermore, complement activation by the classical and by the alternative pathways were demonstrated. Complement aberrations were more pronounced in relapsing otitis than in non relapsing otitis. C1q binding substances, that might possibly cause the complement aberrations found, were present in most of the patients.

Keywords: Otitis media, Complement activation, C1q binding, S.pneumoniae, H.influenzae

INTRODUCTION

As is well known S.pneumoniae is the most frequent agent, in acute purulent otitis media (OME) in children, followed by H.influenzae (cf Klein, 1980). About 20 per cent of children less than 2 years of age with OME have recurring otitis within one month after the onset of a primary episode. The relapses are mostly due to pneumococci (Kamme et al., 1970).

The virulence of microorganisms is partly dependent on their ability to resist phagocytosis in the host. To ensure phagocytosis the encapsulated pneumococcus has to be opsonised by serum components. Pneumococci are ingested slowly by granulocytes and macrophages in the presence of specific capsular antibodies (Ward & Enders, 1933). After binding of complement factor C3b, which is the most important opsonin of invasive pneumococci, to their cell surface, the rate of ingestion is markedly enhanced (Johnston et al., 1969; Shin et al., 1969).

C3b is generated on activation of the complement system (C) (cf Fearon & Austen, 1978). The complement system is comprised of a series plasma proteins which can be activated by immune complexes, but also by other mechanisms. The first factor of the classical pathway, C1, is a macromolecule composed of three subcomponents, C1q, C1r and C1s, held together by Ca^{2+} . C1q binds to antigen-antibody complexes thus activating C1r and C1s. Propagations of C activation leads to generation of C3b. C3b can also be formed through activation of the alternative pathway. In the alternative pathway C3, factor B, factor D and properdin are involved in the activation instead of the early classical pathway components C1, C2 and C4 are (Müller-Eberhard & Götze, 1972).

Disorders within the complement system can contribute to relapsing infections (Abrahamson et al., 1971; Alper et al., 1972). Aberrations in the early factors of the classical pathway have been reported in children, but not adults, with purulent pneumococcal otitis (Johnson et al., 1977; Prellner, 1981a) and in children with serous or mucoid OME (Laurell et al., 1979).

In the present study levels of C1q, C1s, C3, C4, factor B, properdin and C1 subcomponent complexes were determined in acute and convalescent sera from children with relapsing and non-relapsing OME due to S.pneumoniae or H.influenzae. C1q binding substances were also estimated in order to investigate possible interference with the complement system in OME.

MATERIAL AND METHOD

Patients. Eleven patients (mean age 2.8 years) with acute purulent OME due to H.influenzae and 16 patients (mean age 2.8 years) with acute pneumococcal OME were investigated. The complement profiles in 14 of these children have earlier been reported (Johnson et al., 1977) but quantitation of C1 subcomponent complexes and of C1q binding substances was not performed.

H.influenzae or S.pneumoniae were isolated in pure culture from aspirated middle ear effusions. Six children with OME due to H.influenzae and 11 patients with pneumococcal OME had experienced at least one episode of acute otitis during the 4 weeks just prior to the currently investigated OME attack and were thus considered as relapsing cases.

Serum and EDTA-plasma (Na_2EDTA 5mmol/l) samples were obtained on admission and in 21 patients 2 to 3 weeks later. All samples were frozen within 6 hours of sampling and stored in aliquots at -80°C until analysed. Some samples were thawed up to 3 times due to lack of aliquots.

Quantitation of complement components. Levels of C1q, C1s, C3, C4, factor B and properdin were measured by electroimmunoassay (Laurell, 1972), as earlier described (Sjöholm, 1975; Laurell et al., 1978a). The values were given as percentage of the concentration in a pool of normal adult sera (Sjöholm, 1975).

C1 subcomponent complexes. Complexes of C1r-C1s-C1 inactivator (C1r-C1s-C1 IA) and of proenzyme C1r-C1s were demonstrated by crossed immunoelectrophoresis (Laurell et al., 1976). C1r-C1s-C1 IA complexes were quantitated immunochemically (Laurell et al., 1979). In normal sera the levels of C1r-C1s-C1 IA complexes ranged between 11 and 25 per cent of a standard reference (Laurell et al., 1979). C1r-C1s complexes were also quantitated immunochemically as described by Laurell et al., (1981). The levels of C1r-C1s complexes were given as the content of C1r in the complex and expressed in percentages of normal serum levels of C1r (Laurell et al., 1981). By crossed immunoelectrophoresis, C1r-C1s complexes are only rarely demonstrated in normal serum (Sjöholm, 1979; Laurell et al., 1979). The amounts of C1r-C1s complexes detectable by crossed immunoelectrophoresis were equal to or greater than 8 per cent of the reference standard.

C1q binding substances were examined by the C1q deviation test, C1q DT (Sobel et al., 1975) and by the C1q binding assay, C1q BA (Zubler et al., 1976). Values for C1q binding substances were expressed as equivalents of aggregated IgG in ug per ml of serum according to Berglund et al. (1980). Reference curves based on the addition of aggregated IgG in various amounts to fresh normal serum were made for each experiment. In normal sera, C1q binding substances were seldom demonstrable and only occasionally amounts higher than 100 ug per ml were found (Laurell et al., 1979; Berglund et al., 1980).

C-reactive protein (CRP) and α_1 -antichymotrypsin were quantitated by electroimmunoassay (Laurell, 1972). Values below 0.012 and 0.6 g per l, respectively, were considered normal.

Statistical methods. Students t-test for unpaired data and the Wilcoxon test were used.

RESULTS

Complement factor levels.

For relapsing OME the levels of C1q, C1s, C4, C3, factor B and properdin, in samples obtained on the admission, are provided in table 1. The C1q levels were low in relapsing OME both due to pneumococci and to H.influenzae with mean values of 69 and 67 per cent of a normal standard, respectively, C1s levels above the upper normal limit were present in 3 patients. When expressing C1s and C1q in per cent of normal adult values, numerical differences of $62 \pm 43(1SD)$ and 42 ± 39 were found for pneumococcal and H.influenzae OME, respectively.

These differences are significantly ($p, p < 0.0005$) larger than in healthy children (Laurell et al., 1979).

Properdin levels were lowered in both groups to a mean of 83 per cent for pneumococcal OME and 75 per cent for OME caused by H.influenzae. The values of C1q and properdin are significantly lower than those found in healthy children (Laurell et al., 1979), $p < 0.001-0.0005$. The levels of C3 were increased in 5 and factor B in 10 of the patients with relapsing pneumococcal or H.influenzae otitis (table 1). The complement factor levels observed in relapsing OME due to H.influenzae did not significantly differ from those found in OME due to pneumococci.

In non-relapsing OME the complement factor levels were also alike in both etiological groups (table 2). Acute phase serum levels of C1q, C1s, C3, C4 and properdin did not differ significantly from values obtained in healthy children (Laurell et al., 1979). However, 2 of 5 patients with a single pneumococcal OME episode had C1q levels below the lower normal range and also some of the C1s, C3, factor B and properdin levels fell outside the normal ranges (table 2). The C1s level was higher than the corresponding C1q level in all but one serum, with mean values $\pm 1SD$ for the C1s - C1q discrepancy of 24 ± 17 for pneumococcal and 35 ± 27 for H.influenzae OME. These values differ more than those obtained in healthy children, $p < 0.05$ and $p < 0.005$, respectively.

In follow up serum samples most of the complement factor levels tended to normalize. However, C1q levels in relapsed cases were still low with mean values of 74 per cent for pneumococcal and 82 per cent of a normal standard for H.influenzae OME (table 3).

C1 subcomponent complexes

The mean values of C1r-C1s-C1 IA complexes did not differ significantly between relapsing and non-relapsing OME. About half of the patients demonstrated C1r-C1s-C1 IA complexes in quantities exceeding the upper normal limit (table 4).

C1r-C1s complexes, studied by crossed immunoelectrophoresis, were demonstrated in almost all the investigated patients (table 4). The mean amounts of C1r-C1s complexes were greater in relapsing cases than in non relapsing cases, $p < 0.02$. No significant difference was found between OME due to H.influenzae or to pneumococci.

The amount of C1r-C1s-C1 IA found at 2-3 week follow up had decreased in 9 and the amounts of C1r-C1s complexes in 12 of the patients. However, increased levels of the C1 subcomponent complexes were also observed among the patients studied.

C1q binding substances

C1q binding substances were measured by C1q DT and by C1q BA. The variations in values were large and discrepancies in results between the two assays used were evident in some patient.

C1q binding substances, estimated by C1q DT, were present in the acute phase in amounts exceeding 100 ug per ml in more than 50 per cent of patients with pneumococcal OME, both in relapsing and non relapsing cases (table 5). In OME due to H.influenzae 1 patient of 6 with relapsing otitis and 1 patient of 3, without relapses, showed levels of C1q binding substances greater than 100 ug per ml. The levels of C1q binding substances were significantly higher ($p < 0.02$) in patients with pneumococcal OME than in patients with OME due to H.influenzae.

Using C1q BA values above 100 ug per ml were demonstrated in 3 patients.

At follow up, the levels of C1q binding substances, as estimated with C1q DT, were decreased in 10 patients and increased in 6.

Sera containing C1q binding substances in quantities exceeding 100 ug per ml, assessed by C1q DT, showed significantly higher levels of C1r-C1s complexes than sera containing less C1q binding substances ($p < 0.05$). However, C1r-C1s complexes were even found in sera where C1q DT was negative. Furthermore, in 2 sera containing more than 100 ug per ml of C1q binding substances no C1r-C1s complexes could be demonstrated.

C-reactive protein varied from less than 0.012 to 0.126 g per l with median value of 0.026 and was increased in the acute phase of pneumococcal OME in 10 of 16 patients. Increased CRP were seen both in patients with relapsing OME and in patients with a single episode. No increase of CRP was noted in the patients with acute OME due to H.influenzae. α_1 -antichymotrypsin was above normal level (0.6 g/l) in 14 of 16 patients with pneumococcal OME and in 5 of 11 patients with OME due to H.influenzae. Median values were 0.80 and 0.50 g per l, respectively.

At follow up CRP levels had decreased to normal range in all patients, while α_1 -antichymotrypsin often remained slightly elevated.

DISCUSSION

Normal or slightly increased serum levels of C1s, C3, C4 and factor B have earlier been reported in OME (Johnson et al., 1977; Bernstein et al. 1978; Laurell et al., 1979). The increased levels of factor B in particular but also of C1s and C3 observed in many of our investigated patients may be interpreted as an acute phase reaction (Schutte et al., 1974). In the present study the inflammatory plasma protein response was demonstrated by increased levels of α_1 -antichymotrypsin in OME both due to H.influenzae and S.pneumoniae. Furthermore, CRP was usually elevated in pneumococcal OME.

Activation of the classical pathway, indicated by increased amounts of C3r-C3s-C3 IA complexes (Laurell et al., 1978b) was demonstrated as frequently in non relapsing as in relapsing otitis. Involvement of the alternative pathway for C activation, as demonstrated by decrease in properdin, was observed only in relapsed cases. No differences were found, in these respects, between OME due to S.pneumoniae or to H.influenzae.

The most important biological activator of the classical pathway is immune complexes (Augener et al., 1971). The high levels of C1q BA in some patients, indicated presence of circulating immune complexes (Zubler et al., 1976). Specific antibodies are usually present in children with OME, both in serum and in middle ear effusions (Sloyer et al., 1974; Sloyer et al., 1975), and together with the bacterial antigens present, at least in middle ear fluid, they constitute a potential source of circulating immune complexes. It has been shown that addition of different pneumococcal antigens to normal serum can give rise to C1 activation (Prellner, 1981b). Aside from immune complexes, other agents including proteolytic enzymes (Ratnoff & Naff, 1967; Laurell et al., 1978b) and complexes containing C-reactive protein (Volanakis & Kaplan,

1971; Kaplan & Volanakis, 1974; Siegel et al., 1975; Laurell et al., 1978b; Prellner, 1981b) may have accounted for the classical pathway activation.

The alternative pathway may be activated without the initial antigen-antibody complex required by the classical pathway. It can be initiated by a variety of substances including whole pneumococci (Forsgren & Quie, 1974; Fine, 1975; Giebink et al., 1977; Stephens et al., 1977; Prellner, 1979), pneumococcal polysaccharides (Winkelstetlin & Tomasz, 1978; Prellner, 1981b) and lipopolysaccharides from Gram-negative bacteria (Pillemer et al., 1955; Gewurz et al., 1968; Marcus et al., 1971). It might be speculated that excess of bacterial polysaccharides as compared to antibodies is responsible for alternative pathway activation demonstrated in relapsing otitis.

Usually a close correlation exists between serum levels of C1q, C1r and C1s (Sjöholm, 1975). Reduced C1q as compared to C1s was almost regularly present in children with otitis, both due to pneumococci (Johnson et al., 1977) and to H.influenzae. However, the C1q, C1s discrepancy was far more pronounced in relapsing than in non relapsing OME. The appearance of C1r-C1s complexes, demonstrated in most of the investigated sera and with the highest quantities found in relapsing cases, could be ascribed to the disproportion between levels of the C1 subcomponents.

Substances capable of binding C1q were demonstrated in half of the sera. Different possibilities for interference with the C system exist for these substances. When the C1q binding is due to immune complexes, classical pathway activation results. C1q binding substances, could also represent bacterial components, capable to bind

C1q without the participation of antibodies (Sobel et al., 1975). Intact H.influenzae and S.pneumoniae (Prellner, 1980) as well as pneumococcal cell wall components have antibody independent C1q binding capacity (Prellner, 1981b). Pneumococcal polysaccharides are capable of binding C1q in vitro without activation of the C system (Prellner, 1981b). This mechanism may account for the decreased levels of C1q demonstrated in otitis. The C1q binding capacity found in OME sera could partly be due to the presence of C1r-C1s complexes, but it should be emphasized that C1q binding substances appeared also in sera devoid of or with low amounts of C1r-C1s complexes.

The C aberrations earlier found in children with acute pneumococcal OME (Johnson et al., 1977) were not restricted to disease caused by S.pneumoniae. Children with OME due to H.influenzae had the same serum C profile. The changes in C components observed in OME were more pronounced in relapsing cases than in children suffering from a single attack. The reduced C1q levels and the presence of C1r-C1s complexes indicates disturbed function of the classical pathway which may lead to suboptimal generation of the important opsonin C3b and thus contribute to relapses.

Table 1

Levels of complement factors, in per cent of normal standard, obtained on admission in 17 patients with relapsing purulent otitis media (OME). Eleven OME episodes were due to S.pneumoniae (pnc) and 6 to H.influenzae (H.i.). P stands for properdin and B for factor B.

		frequency of values				
		mean	range	below normal range	above normal range	normal range
C1q	pnc	69	29- 97	6/11	0	78-130
	H.i.	67	6-110	4/ 6	0	
C1s	pnc	131	88-198	0	2/11	79-139
	H.i.	110	85-146	0	1/ 6	
C3	pnc	120	85-153	0	2/11	70-136
	H.i.	127	73-170	0	3/ 6	
C4	pnc	142	68-200	0	0	53-207
	H.i.	115	60-152	0	0	
B	pnc	163	116-200	0	7/11	59-154
	H.i.	142	110-173	0	3/ 6	
P	pnc	83	44-118	1/11	0	57-153
	H.i.	75	50-106	2/ 6	0	

Table 2

Levels of complement factors, in per cent of normal standard in acute sera in 10 patients with non relapsing purulent otitis media (OME). Five OME episodes were due to S.pneumoniae (pnc) and 5 to H.influenzae (H.i.) P stands for properdin and B for factor B.

		mean	range	frequency of values		
				below normal range	above normal range	normal range
C1q	pnc	85	57-120	2/5	0	78-130
	H.i.	99	84-118	0	0	
C1s	pnc	109	91-119	0	0	79-139
	H.i.	134	110-118	0	2/5	
C3	pnc	112	87-142	0	1/5	70-136
	H.i.	121	96-142	0	1/5	
C4	pnc	124	84-182	0	0	53-207
	H.i.	109	73-171	0	0	
B	pnc	121	96-151	0	0	59-154
	H.i.	147	107-200	0	2/5	
P	pnc	108	75-190	0	1/5	57-153
	H.i.	91	71-135	0	0	

Table 3

C1q, C1s and factor B levels (mean $\pm$ 1SD) in acute and follow-up sera from patients with relapsing purulent otitis media due to S.pneumoniae (n=11) and H.influenzae (n=6).

	<u>S.pneumoniae</u>		<u>H.influenzae</u>	
	first	follow-up	first	follow-up
	sample	sample	sample	sample
C1q	69 $\pm$ 24	74 $\pm$ 18	67 $\pm$ 33	82 $\pm$ 22
C1s	131 $\pm$ 27	120 $\pm$ 14	110 $\pm$ 22	119 $\pm$ 14
factor B	163 $\pm$ 25	135 $\pm$ 34	142 $\pm$ 21	103 $\pm$ 25

Table 4

Quantities of C1r-C1s-C1 IA and C1r-C1s complexes in per cent of reference standards (see materials and methods) in acute sera in patients with purulent otitis media (OME). Seventeen OME were relapsed cases and 10 were not. Causative agents were S.pneumoniae (pnc) or H.influenzae (H.i.).

hihg= values above the upper normal limit

n= number of patients

		C1r-C1s-C1 IA complexes			C1r-C1s complexes		
		mean	range	high n	mean	range	high n
RELAPSING OME	pnc	25	17-36	5/11	19	6-35	10/11
	H.i.	22	12-31	2/6	16	3-32	5/6
NON RELAPSING OME	pnc	26	15-38	3/5	9	2-13	3/5
	H.i.	22	17-29	2/5	10	3-15	4/5

Table 5

Quantities of C1q binding substances, estimated with C1q deviation test, in acute sera in patients with purulent otitis media (OME) due to S.pneumoniae (pnc) and H.influenzae (H.i.).

		mean	range	frequency with levels > 100 ug/ml
RELAPSING	pnc	145	55-350	6/11
OME	H.i.	79	0-230	1/6
NON	pnc	144	50-320	3/5
RELAPSING	H.i.	90	0-170	1/3
OME				

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Zusammenfassung

Die Komplement-Komponenten C1q, C1s, C3, C4, Faktor B und Properdin wurden zusammen mit den Komplementen der C1-Sub-Komponenten und C1q bindenden Substanzen in akuten und in rekonvaleszenten Proben von Patienten mit recidivierender und nicht-recidivierender Otitis Media, verursacht von S.pneumoniae und H.influenzae, gemessen.

Die Analyse dieser Komplexe zusammen mit dem Befund niedriger C1q Niveaus wies auf eine gestörte Funktion bei akuter OME. Ausserdem wurde die Komplement-aktivierung durch den klassischen und durch den alternativen Aktivierungsweg demonstriert.

Veränderungen des Komplementprofils waren ausgeprägter in recidivierender als in nicht-recidivierender Otitis.

C1q-bindende Substanzen, die diese Veränderungen verursachen könnten, wurden in den meisten Patienten nachgewiesen.

IMMUNE COMPLEXES AND COMPLEMENT IN SEROUS AND MUCOID OTITIS MEDIA

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Abstract. The occurrence and quantity of immune complexes in middle ear effusion (MEE) and serum, as well as serum levels of complement (C) factors were investigated in patients with chronic otitis media. Immune complexes were demonstrated in 85% of the serous MEE and in 28% of the sera. Depressed C1q values and presence of abnormal complexes, composed of sub-components of the first C factor, indicated a disturbed function of the C system. Activation of C by the classical pathway was demonstrated in 23% of the patients. Decreased levels of properdin were also noted. The disorders within the C system tended to normalize as the otitis subsided.

The etiology of chronic serous or mucoid otitis media (OME) is still not clarified. Bacterial cultures from the middle ear effusions have most often proved negative (Liu et al., 1976; Giebink et al., 1979). The concept that chronic serous otitis media is immunologically mediated was suggested by Veltri & Sprinkle (1976). In one study the presence of immune complexes in middle ear effusions has been reported (Maxim et al., 1977).

Complement (C) is a well defined system of plasma proteins, which includes several unique enzymes. C is involved in the defence against invading microorganisms and in inflammation. Complement is activated by immune complexes but also by other mechanisms. In the classical pathway of C activation the first factors of complement (C1q, C1r, C1s) are bound and activated by antigen-antibody complexes. If this occurs on a bacterial cell the complement cascade engages C3 which in its activated form, C3b, is bound to

the cell surface. It has been shown that pneumococci are taken up by polymorphonuclear leukocytes mainly when coated with antibody and complement and that opsonization and phagocytosis of pneumococci are essentially dependent on C3b bound to the bacterial cell (Winkelstein et al., 1975).

Disorders within the complement system have been reported in children with relapsing otitis media due to pneumococci (Johnson et al., 1977). Decreased C1q together with normal or elevated levels of C1r and C1s were found in 60% of these patients. In addition increased amounts of complexes composed of C1 subcomponents, C1r-C1s and C1r-C1s-C1 inactivator (C1r-C1s-C1 I A) were a constant finding.

In order to elucidate mechanisms contributing to the chronic course in serous or mucoid otitis media, studies were undertaken concerning the occurrence and quantity of immune complexes in middle ear effusion (MEE) and serum and the pattern of some of the complement components was analysed.

MATERIALS AND METHODS

Patients

Eighty-six patients (aged 1-8 years, mean: 3.9) with chronic otitis media with effusion (OME), serous or mucoid, were investigated. The duration of OME was between one and 6 months. Myringotomy was performed under

Table 1. Bacteriological findings in nasopharyngeal samples in patients with chronic otitis media and from healthy children

Bacteria	Number of individuals harbouring bacteria	
	Patients (n=86)	Healthy individuals (n=29)
<i>S. pneumoniae</i>	35	3
<i>H. influenzae</i>	21	7
Gr. A streptococci	2	1
<i>B. catarrhalis</i>	34	22
No pathogens isolated	14	10

general anesthesia in all patients. For comparison, 29 children aged between 2 and 10 years (mean: 6.4) were also investigated. The samples were obtained under general anesthesia. The control group comprised children under surgical treatment for hernia inguinalis, phimosis, naevus, or short frenulum linguae.

Middle ear effusions were collected from 20 patients with serous OME by aspiration through the ear drum.

Serum and EDTA-plasma (Na_2EDTA 5 mmol/l) were obtained at the time of the first myringotomy, and again in 31 patients 4 weeks later. Serum, plasma and middle ear effusions were frozen within 3 hours of sampling and stored in aliquots at -80°C until analysed.

Bacteriological examination

Specimens were obtained from nasopharynx using a sterile cotton swab and transported to the Bacteriology Department in haematin agar tubes within 24 hours. The nasopharyngeal specimens were inoculated on haematin agar and blood agar and the plates incubated at 37°C for 18 hours in 6% CO_2 atmosphere and anaerobically (Gas Pak® System) respectively.

Pneumococci, β -hemolytic streptococci and *H. influenzae* were identified using standard methods. Streptococci were serologically grouped by coagglutination (Christensen et al., 1973). *B. catarrhalis* was identified as described by Kamme (1970).

Serum IgG was quantitated according to Grubb (1970). The normal ranges used for the different age groups were those detailed by Johansson & Berg (1967).

C-reactive protein was estimated by electroimmunoassay (Kindmark, 1969).

Quantitation of complement components

Levels of C1q, C1s, C3, C4, and properdin were measured by electroimmunoassay (Laurell, C.-B., 1972; Laurell, A.-B. et al., 1978). The normal ranges referred to are those given for adults (Sjöholm, 1975). Children, aged between 2 and 10, have similar levels of C1q. However, C3 levels in this age group are approximately 10% higher when compared with adults (Yonemasu et al., 1978).

C1 subcomponent complexes. Complexes of C1r-C1s and C1r-C1s-C1 IA, respectively, were demonstrated by crossed immunoelectrophoresis (Laurell et al., 1976).

C1r-C1s-C1 IA complexes were quantitated immunochemically (Laurell et al., 1979). The normal range of C1r-C1s-C1 IA was 11–25% of a reference serum (Laurell et al., 1979).

C1q binding substances were examined by the C1q deviation test C1q DT (Sobel et al., 1975) and by the C1q binding assay, C1q BA (Zubler et al., 1976). Values were expressed in equivalents of heat aggregated IgG ($\mu\text{g/ml}$). For each experiment, calibration curves were prepared by adding various amounts of aggregated IgG to fresh serum.

Statistical methods

Student's *t*-test for paired and unpaired data and the χ^2 -test were used.

RESULTS

I. Comparison between patients with chronic OME and healthy children

Bacteriological findings. *S. pneumoniae*, *H. influenzae*, group A streptococci and *B. catarrhalis* were isolated from the nasopharyngeal samples in 72 of 86 (84%) of the OME patients and in 19 of 29 (66%) of the

Table II. Complement factor levels in sera from 86 patients with chronic OME and from 29 healthy children

Complement factor	Mean value $\pm$ 1 S.D. in		Statistical difference in mean value
	patients	healthy children	
C1q	82 $\pm$ 14 %	86 $\pm$ 15 %	No difference ($p>0.05$)
C1s	112 $\pm$ 17 %	97 $\pm$ 15 %	No difference ($p>0.05$)
C1s-C1q ^a	20 $\pm$ 16	11 $\pm$ 15	Significant difference ($p<0.02$)
C3	107 $\pm$ 16 %	98 $\pm$ 21 %	Significant difference ($p<0.05$)
C4	99 $\pm$ 33 %	95 $\pm$ 30 %	No difference ($p>0.05$)
Properdin	85 $\pm$ 17 %	105 $\pm$ 28 %	Significant difference ($p<0.001$)

^a Numerical difference between C1s and C1q levels in individual sera.

control patients (Table I). Thirty-one of all samples harboured more than one pathogen.

Immunoglobulin G levels were within normal ranges. No increase in C-reactive protein (i.e. less than 12 mg/l) was found.

Levels of complement component in serum. The mean C1q and C1s values of sera from OME patients did not differ from those of the controls ($p>0.05$), (Table II). However, C1q was disproportionately low compared with C1s in individual sera from most of the OME patients. Compared with the control group the mean values for this numerical difference between C1s and C1q was statistically significant ($p<0.02$, Table II).

C4 values did not differ between the two groups (Table II). C3 were within the normal ranges in all sera, but the mean value was higher in the OME patients than in control sera ($p<0.05$).

The properdin values were lower in sera from OME patients than in sera from the control group ($p<0.001$, Table II).

C1 subcomponent complexes. Increased amounts of C1r-C1s-C1 IA complexes were found in 23% of sera from OME patients. The levels of C1r-C1s-C1 IA complexes were within the normal range in all sera from the control group (Table III). This difference was statistically significant ($p<0.025$).

C1r-C1s complexes were detected in 41% of the OME patients and in 10% in the control group ($p<0.01$, Table III).

C1r-C1s complexes were found more fre-

quently in sera where a discrepancy between C1s and C1q values was present (Fig. 1). C1r-C1s complexes were present in 32 of 72 (46%) patient sera from whom potentially pathogenic bacteria were isolated and in 3 of 14 (21%) sera from patients without such bacteria (Table IV). This difference was not statistically significant ($p>0.05$).

C1q binding substances. Fifty-seven sera from patients with chronic OME were tested for C1q binding substances. With C1q DT sera from OME patients showed significantly higher amounts of C1q binding substances than sera from the controls. C1q reacting substances corresponding to more than 100 μ g aggregated IgG per ml were present in 16 of 57 sera from OME patients and in 1 of 29 sera in the control group ($p<0.01$). Occurrence of

Table III. Frequency of C1r-C1s complexes and increased levels of C1r-C1s-C1 IA complexes in sera obtained from 86 patients with chronic OME and from 29 healthy children

	Frequency of C1 subcomponent complexes in sera of	
	patients	healthy children
Increased levels of C1r-C1s-C1 IA complexes ^a	20/86	0/29
Presence of C1r-C1s complexes	35/86	3/29

^a More than 25% of a reference serum. See Materials and Methods.

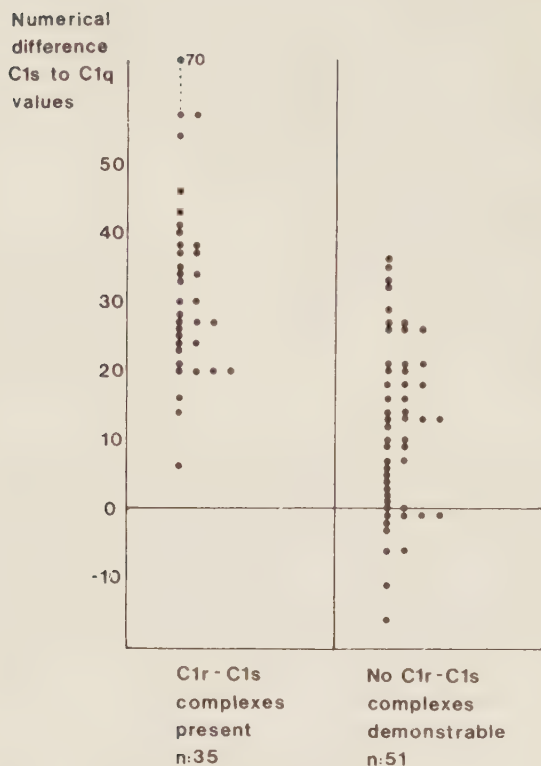


Fig. 1. Numerical differences between C1s and C1q serum concentrations in chronic OME patients in relation to presence of C1r-C1s complexes.

C1q binding substances in patient sera could not be correlated to presence of pathogens in the nasopharyngeal specimens ($p > 0.05$, Table IV).

When the same sera were tested for C1q binding substances using the C1q BA, there was no difference between controls and OME patients.

C1q binding substances, estimated by C1q DT, were present in 32% of the OME sera containing C1r-C1s complexes as compared with 19% of the OME sera where no such complexes were present. This difference was not statistically significant, $p > 0.05$.

In serous MEE, C1q binding substances detected by C1q DT and/or C1q BA were found in 17 of 20 samples tested. These values ranged from 130 $\mu\text{g/ml}$ to $>2000 \mu\text{g/ml}$.

In addition, C1q binding substances were demonstrated in the serum of 6 of these 20

patients and only when such substances were present in MEE.

The presence of C1q binding substances in MEE could not be correlated to the occurrence of pathogenic bacteria in the nasopharynx. High levels of C1q reactive substances (540 $\mu\text{g/ml}$, respectively $>2000 \mu\text{g/ml}$) were demonstrated in 2 patients where no pathogens were isolated from the nasopharynx.

II. Follow-up examination of patients with chronic OME

C1 subcomponent complexes and C1q binding substances. Blood was sampled from 31 patients who were re-investigated 4 weeks after the first myringotomy and aspiration of MEE. When microscopic otoscopy demonstrated retracted tympanic membranes and/or MEE, a second myringotomy was performed.

MEE was again present in 11 patients (group I). The other 20 patients did not demonstrate MEE (group II), although some of them had retracted tympanic membranes. In both groups, C1r-C1s complexes and increased amounts of C1r-C1s-C1 IA complexes were less frequently found in the follow-up serum samples (Table V). In the patients without MEE at follow-up, however, the decrease in C1r-C1s-C1 IA complexes was most pronounced ($p < 0.001$).

At follow-up, the C1q DT revealed unchanged serum levels in 41%, increased levels

Table IV. Occurrence of C1r-C1s complexes and C1q binding substances in sera correlated to bacteriological finding in nasopharynx in patients with chronic OME

Bacteria in nasopharynx	Frequency of C1r-C1s complexes	C1q DT ^a $>100 \mu\text{g/ml}$
<i>S. pneumoniae</i>	6/9	2/5
<i>H. influenzae</i>	9/18	4/15
<i>B. catarrhalis</i>	9/18	6/13
More than one pathogen	8/27	3/18
No pathogens	3/14	1/6

^a Estimated with C1q DT. Levels greater than 100 $\mu\text{g/ml}$ were regarded as positive.

Table V. Frequency of C1r-C1s complexes and elevated levels of C1r-C1s-C1 IA complexes in sera from patients with chronic OME, at first myringotomy and at the 4-week follow-up

	C1r-C1s complexes		C1r-C1s-C1 IA complexes	
	First sample	Follow-up sample	First sample	Follow-up sample
Group I (MEE present at follow-up)	6/11	3/11	4/11	3/11
Group II (MEE absent at follow-up)	8/20	4/20	8/20	1/20

in 37 % and decreased levels in 22 % of the patients.

DISCUSSION

The mean ages for patients in the OME group (3.9 years) and the control group (6.4 years) did differ ($p<0.05$). However, all the differences described between these groups remained unaltered, when only OME patients 4 years or older ($n=47$; mean 5.9 years) were included.

The presence of C1r-C1s-C1 IA complexes in normal sera indicates C1 activation (Laurell et al., 1978). The elevated levels of such complexes in serum from patients with chronic OME implies increased C1 activation also in the absence of manifest hypocomplementemia. C1 can be activated by immune complexes, by CRP in complex with various poly anion/cations (Siegel et al., 1975) and with pneumococcal C-polysaccharide (Kaplan & Volanakis, 1974; Claus et al., 1977) and by enzymes such as plasmin and trypsin (Ratnoff & Naff, 1967). The present investigation does not, however, elucidate the nature of the C1 activating substances appearing in OME which are evidenced by increased C1r-C1s-C1 IA level.

An impressive finding was the high amounts of C1r-C1s complexes present in 41 % of patients with chronic OME, the information of

which has not yet been explained. The relative excess of C1r and C1s compared with C1q may be the result of an acute phase reaction. However, normal levels of the acute phase protein CRP, registered in OME patients, does not sustain this possibility. A dissociation equilibrium of C1 subcomponents in normal serum has recently been reported (Bartholomew & Esser, 1977). As it is known that some bacterial components bind C1q directly (Loos et al., 1974), it can be speculated that such binding may cause a disequilibrium between the C1 subcomponents, which leads to the formation of C1r-C1s complexes.

C1q binding substances exceeding 100 $\mu\text{g/ml}$ were demonstrated in the sera of 28 % of patients by C1q DT, although the C1q BA was negative. Various bacterial products may be detected by the C1q DT (Sobel et al., 1975), but probably not by the C1q BA (Zubler et al., 1976), whereas immune complexes are detected by both tests (Sobel et al., 1975; Zubler et al., 1976). Thus the C1q binding capacity demonstrated in OME sera is possibly not due to immune complexes but other C1q reactive substances. High amounts of C1q binding substances were detected in MEE using both the C1q BA and C1q DT, indicating the presence of immune complexes.

Maxim et al. have demonstrated immune complex-like substances in serous MEE and proposed that they were composed of anti-

bodies and bacterial antigens derived from the nasopharynx (1977). In the present investigation, immune complexes were demonstrated in MEE from 2 patients where no pathogenic bacteria could be isolated from the nasopharynx. This finding does not, however, exclude that antigens participating in the formation of immune complexes are or have been present in the nasopharynx.

At the 4-week follow-up all but one of the patients in whom MEE was not present displayed C1r-C1s-C1 IA levels within normal range, while C1r-C1s complexes had disappeared in 4 of 8 patients. It was, however, not possible in the present study to demonstrate in sera correlation between decreased concentrations of C1q binding substances and of C1r-C1s-C1 IA complexes or the disappearance of C1r-C1s complexes.

Besides activation of C by the classical pathway, we have also found decreased levels of properdin in chronic OME patients which may indicate that complement is activated by the alternative pathway as well. No further attempts were made in the present study to search for other parameters to support this latter possibility.

In conclusion, the presence of immune complexes in MEE and of C1q reactive substances in serum supports the concept that the inflammatory process in chronic otitis media may be sustained by an immunological process.

ZUSAMMENFASSUNG

Das Auftreten und die Quantität von Immunkomplexen im Mittelohrexsudat und Serum sowie das Serumniveau des Komplements bei Patienten mit chronischer Otitis media wurden untersucht. Immunkomplexe wurden in 85 % in serösem Mittelohrexsudat und in 28 % in den Sera nachgewiesen. Verminderte C1q-Werte und das Vorkommen von anormalen Komplexen, zusammengesetzt von Untereinheiten des ersten C-Faktors, hat auf eine gestörte Funktion des Komplement-Systems hingewiesen. Erhöhte C-Aktivierung durch den klassischen Aktivierungsweg wurden bei 23 % der Patienten nachgewiesen. Vermindertes Niveau von Properdin wurde auch beobachtet. Nach Abklingen der Otitis hatten die Veränderungen des Komplement-Systems eine Tendenz zur Normalisierung.

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Complement in Pneumococcal Infections with Varying Degrees of Severity

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ABSTRACT. Complement component levels (C1q, C1s, C4, C3, factor B and properdin) and C1 subcomponent complexes (C1r-C1s, C1r-C1s-C1 inactivator, IA) were studied in 16 adults with pneumococcal infections of varying severity. Patients with fulminant disease and signs of septic shock showed pronounced hypocomplementemia. In patients with pneumococcal pneumonia or meningitis elevated levels of C1r-C1s-C1 IA complexes indicated activation of C1, despite normal levels of C1q, C1s, C4 and C3. Moderately decreased properdin values suggested involvement of the alternative pathway. In adults with pneumococcal otitis no changes in the complement profile was found. In contrast, pronounced aberrations of the C1 subcomponents were earlier demonstrated in children with otitis.

INTRODUCTION

It has earlier been reported that phagocytosis of pneumococci occurred only slowly in the presence of heated immune serum and that addition of complement markedly enhanced the rate of ingestion (12, 26, 31). On complement activation via the classical or alternative pathways C3b is formed by cleavage of native C3 (23). C3b is an important opsonic factor in the defence against i.a. pneumococcal infections, which has been well documented (1, 2).

Complement activation and consumption is generally found in fulminant infections caused by gram-negative as well as gram-positive bacteria (4, 5, 6, 8, 20).

In less severe pneumococcal infections in adults moderate consumption of the complement factors, C3 and properdin, was found, while the early factors of the classical pathways were essentially normal (3, 9). It was claimed that complement was activated via the alternative pathway (3).

Abnormalities of the C1 subcomponents were constantly found in serum of children with relapsing otitis media due to pneumococci (10). Thus, high concentrations of C1r-C1s-C1 inactivator (IA) complexes, indicative of increased C1 activation (14) were demonstrated. Decreased C1q together with normal or elevated levels of C1s was found in 60% of these children and high amounts of complexes of C1r-C1s was a regular finding.

A functionally intact C1 factor is necessary for activation of the classical pathway. Disorders within the C1 component may possibly impair the important opsonic function of the classical pathway. The pronounced aberrations of the C1 subcomponents found in children with otitis media challenged the present investigation of C factor levels and C1 subcomponent complexes in adults with pneumococcal infections of various severity. Furthermore, the C activation mechanism in pneumococcal infections was studied.

MATERIALS AND METHODS

Patients

The clinical data of 16 patients with pneumococcal infections, 7 females and 9 males aged 17 to 89 years, are summarized in Table I.

Patients with acute otitis media had otalgia and purulent middle ear effusion. Pneumococci were obtained in pure culture from nasopharynx. Patients with pneumonia were admitted to the hospital with fever, purulent sputum and roentgenographic evidence of pulmonary consolidation. In nasopharyngeal cultures pneumococcus was the sole pathogenic bacterial species found. In patients with clinical signs of acute meningitis or septicemia, pneumococci were cultured from cerebrospinal fluid (CSF) and blood, respectively.

In patients 7 and 16 splenectomy had been performed 5 and 2 years prior to pneumococcal infection, respectively. Both patients were regarded as healthy at time of contracting the illness. The other patients had neither splenectomy nor previous history of chronic disease like

Table I. Clinical data of the patients investigated

Pat. no.	Sex	Age (yrs.)	Diagnosis	Pneumococci isolated from	Sera obtained on day	Complications and comments
1	F	23	Otitis media acuta	N	1, 18	None
2	F	27	Otitis media acuta	N	1	None
3	M	17	Otitis media acuta	N	1, 23	None
4	M	17	Otitis media acuta	N	1	None
5	M	30	Otitis media acuta	N	1, 21	None
6	M	31	Otitis media acuta	N	1	None
7	F	37	Meningitis	CSF, B	4, 12, 19	Recovered from Mb Hodgkin. Splenectomized
8	F	69	Meningitis	N, CSF, B	3, 15, 18	Embolia pulmonis on day 19
9	F	72	Meningitis	N, CSF, B	1, 2, 22	None
10	M	29	Meningitis, frontal sinusitis	CSF, B	1, 15	None
11	M	39	Meningitis	N, CSF, B	1, 17	X-ray: pulmonary consolidation day 7-24. Persistent cerebral damage
12	F	78	Pneumonia	N	3, 13	None
13	M	48	Pneumonia	N	4	None
14	M	89	Pneumonia	N	1, 2, 19	None
15	F	48	Pneumonia, septicemia	N, S, B	3, 7, 10, 23	Shock day 2. Recovered.
16	M	77	Septicemia	B	1	Splenectomized. Shock. Fatal outcome

N=nasopharynx; CSF=cerebrospinal fluid; B=blood; S=sputum

e.g. immunodeficiency, autoimmune disease or malignancy.

This study was performed during 1978 and 1979. Patients with acute otitis media were treated at the ENT clinic of Lund, patients 12, 13, and 14 attended the Infectious Clinic of Helsingborg, and the remaining patients were hospitalized at the Infectious Clinic of Lund. Clinical data concerning the patients were gathered from records.

Serum and EDTA plasma (Na_2EDTA , 5 mmol/l)

Samples were obtained during the acute phase in all patients (days 1 to 4 of the infection) and in 11 patients during convalescent phase (days 13 to 23). Serum and plasma were frozen within 3 h of sampling and stored at -80°C until analysed.

C-reactive protein

C-reactive protein (CRP) was quantitated by electroimmunoassay (17). Values below 12 mg/l were considered normal.

Quantitation of complement components

Levels of C1q, C1s, C3, C4, factor B and properdin were measured by electroimmunoassay (17), as earlier described (15, 27). The values were given as percentage of the concentration in a pool of normal sera (27).

C1 subcomponent complexes

Complexes of C1r-C1s and of C1r-C1s-C1 IA were demonstrated by crossed immunoelectrophoresis (13). C1r-C1s-C1 IA complexes were quantitated immunochemically (16). In normal sera the levels of C1r-C1s-C1 IA complexes ranged between 11 and 25% of a standard reference (16).

Specimens obtained in acute and convalescent phase were analysed simultaneously to minimize the error of the methods.

Statistical significance

Statistical significance was assayed by Student's *t*-test for paired data.

RESULTS

C-reactive protein

The CRP levels were increased in the early phase of infection in all but one, no. 16, of the patients with pneumonia, meningitis and/or septicemia. Three patients with otitis media had elevated levels (Table II). The CRP levels of the follow-up samples

Table II. CRP levels in the acute phase in patients with pneumococcal infections

	CRP	
	Mean $\pm$ 1 S.D. (mg/l)	High ^a (N)
Patients with otitis media (N=6)	25 $\pm$ 23	3
Patients with pneumonia meningitis and/or septicemia (N=10)	278 $\pm$ 194	9

^a Values above normal (12 mg/l).

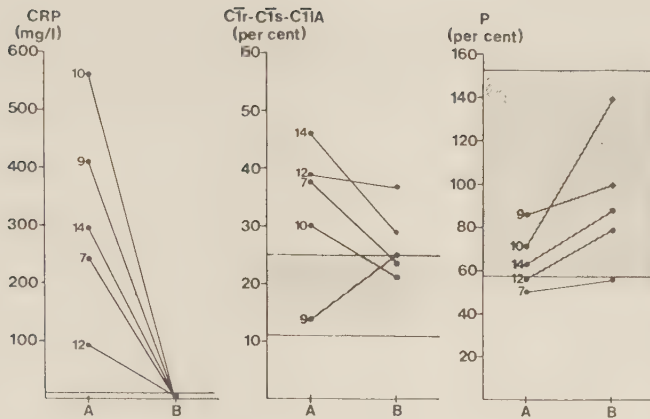


Fig. 1. Concentrations of CRP, C3r-C3s-C3IA complexes and properdin (P) in the acute (A) and follow-up (B) sera in 5 patients with pneumonia or meningitis, healing without complications. C3r-C3s-C3IA levels are given in per cent of standard reference, P in per cent of normal reference. Normal ranges indicated by shadowed areas. Figures at A refer to patient numbers given in Table I.

were returned to normal in all but 2 patients (nos. 8 and 11). These two patients suffered from a prolonged disease. Fig. 1 shows the CRP values in both acute phase and convalescence for 5 patients with pneumonia or meningitis who recovered without complications.

Complement component levels and C1 subcomponent complexes

In the patients with acute otitis media the levels of the complement factors were essentially within normal range (Table III), as were the levels of C3r-C3s-C3IA complexes (mean $\pm$ 1 S.D.: $16 \pm 5\%$ of a standard reference). No C1r-C1s complexes were demonstrated.

The levels of C1q, C1s, C4, C3, factor B and properdin in samples obtained in the acute phase

from patients with meningitis or pneumonia are presented in Table IV. The concentrations of C1q, C4 and C3 were essentially normal. An increase in C1s was found in 4 and in factor B in 6 patients. Although C1s and factor B fell in the follow-up samples, they remained slightly increased (mean $\pm$ 1 S.D. of C1s 123 ± 20 and of factor B 147 ± 32). These values did not differ significantly from these found in the acute phase ($p > 0.05$).

The mean value for properdin in the acute phase in this patient group was $72 \pm 21\%$ (normal range 57–153). Two values fell below the lower limit of the normal range and the others were in the normal range. During convalescence properdin increased (Fig. 1) significantly ($p < 0.05$).

With the exception of one patient (no. 9) C3r-C3s-C3IA levels were increased in the acute phase

Table III. Levels of complement factors, in per cent of normal standard, in acute sera from 6 patients with acute otitis media

	Mean	Range	Low ^a (N)	High ^b (N)	Normal range
C1q	101	88–114	0	0	78–130
C1s	115	94–148	0	1	79–139
C4	120	88–144	0	0	53–207
C3	109	89–120	0	0	70–136
P	112	85–150	0	0	57–153
Factor B	121	60–205	0	1	58–154

^a Values below normal range.

^b Values above normal range.

Table IV. Levels of complement factors, in per cent of normal standard, in acute sera in 8 patients with pneumonia or meningitis

	Mean	Range	Low ^a (N)	High ^b (N)	Normal range
C1q	108	79–134	0	1	78–130
C1s	138	69–177	1	4	79–139
C4	112	56–179	0	0	53–207
C3	115	84–138	0	1	70–136
P	72	50–115	2	0	57–153
Factor B	178	132–220	0	6	58–154

^a Values below normal range.

^b Values above normal range.

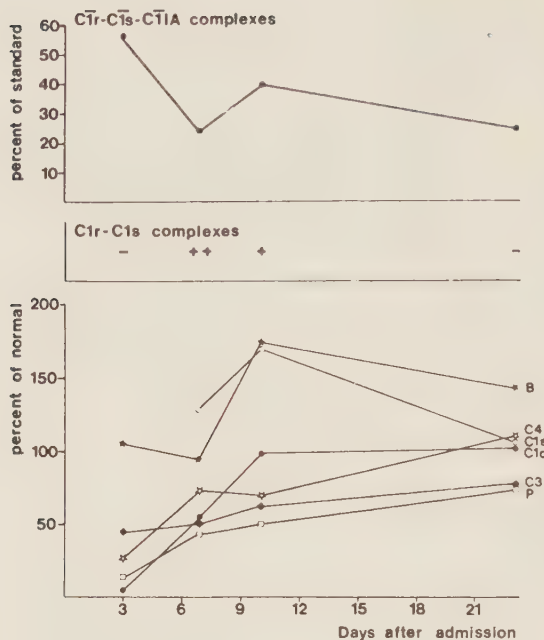


Fig. 2. Levels of complement factors, C1r-C1s-C1 IA complexes and C1r-C1s complexes in a patient (no. 15), who recovered from shock following septicemia. Complement component levels are given in per cent of normal reference, C1r-C1s-C1 IA levels in per cent of a standard reference (ref. 16).

in the patients with pneumonia or meningitis (mean $\pm$ 1 S.D.: 38 ± 7) and decreased on recovery ($p < 0.05$). At the occasions when follow-up samples were taken, 2 patients with meningitis (nos. 8 and 11) suffered from pulmonary complications, embolia pulmonis and pneumonia, respectively, and in both increased levels of C1r-C1s-C1 IA complexes remained. Thus, the levels of C1r-C1s-C1 IA varied from 54 to 48% in patient 8 and from 33 to 48% in patient 11.

C1r-C1s complexes were not demonstrated in the acute or in the convalescent samples from patients with meningitis or pneumonia, except in one patient (no. 9), where a moderate amount of C1r-C1s complexes was found in a second serum sample taken 2 days following admission.

Two patients (nos. 15 and 16) with severe pneumococcal infection got acute circulatory collapse. In these patients a marked hypocomplementaemia was found in connection with the shock. All complement factor levels analysed were less than 25% of a normal reference and no C1r-C1s-C1 IA complexes were found in one (no. 16). This patient died shortly afterwards.

The other patient (no. 15) gradually recovered and the complement levels returned to normal (fig. 2). During 3 weeks following shock C1q increased from $<10\%$ to 101, C4 from 27 to 118, C3 from 45 to 75 and properdin from <25 to 75%. With the technique used it was not possible to determine the C1s level in the acute phase sample because of the diffuse appearance of the C1s precipitate. In the following samples (day 7 and 10) C1s was measured to 128 and 170%, respectively, and reverted to normal (104%) by day 23. Factor B was highest on day 10 (172%) and had dropped to within normal range (142%) on day 23. Elevated levels of C1r-C1s-C1 IA complexes were observed on day 3 and 10, but were within the normal range on day 7 and 23. C1r-C1s complexes were present in the samples obtained on day 7 and 10, but not on day 3 or 23 (Fig. 2).

DISCUSSION

In the present investigation serum complement was studied in patients who had pneumococcal infections with varying degrees of severity.

In agreement with the findings of earlier studies (3, 4, 5, 6, 8, 20), manifest hypocomplementemia was found in patients with fulminant disease exhibiting signs of septic shock. In non-complicated pneumococcal pneumonia or meningitis activation of C1 and decrease in properdin levels were demonstrated. No evidence for complement activation was shown in adults with acute pneumococcal otitis media.

Hypocomplementemia associated with fulminant pneumococcal disease could be related to the development of disseminated intravascular coagulation, as suggested previously (24, 30).

The decrease of properdin, demonstrated in patients with pneumonia or meningitis, indicated involvement of the alternative pathway of the C system and agreed with earlier studies (3, 9, 23). However, the elevated levels of C1r-C1s-C1 IA complexes observed in our patients indicate activation of C1 (14), despite normal levels of the early classical pathway components. Thus, the lowered concentrations of properdin might be induced by activation of the classical pathway maintaining the C3 feed-back mechanism (11, 19), as well as by direct activation of the alternative pathway. Considering the high levels of CRP, it seems likely that consumption of components such as C3 was masked by an acute phase reaction (25). This could also explain the high levels observed for factor B and C1s, since these complement proteins are known to show greatest elevation in the acute phase of inflammation (25).

In contrast to the aberrations of the C1 sub-components in children with otitis media (10), no changes in the complement profile was demonstrated in adults with pneumococcal otitis. This might indicate that pneumococcal otitis is a more pronounced disease in the childhood. C1r-C1s complexes, regularly found in high amounts in children with relapsing otitis, could not be demonstrated in adults with otitis, but were present in 2 patients with more severe pneumococcal infections.

The precise mechanisms by which pneumococci react with the C system in disease are not clear. Components of the bacterial cell might be of importance and reactions of C components with these, either directly or mediated by antibodies must be considered. Intact pneumococci as well as cell wall components have been demonstrated to activate serum complement by the classical and the alternative pathway *in vitro* (7, 21, 28, 32, 33). Direct inter-

actions, independent of antibodies, have been demonstrated between lipopolysaccharides and C1 (18) and between pneumococci and C1q (22). It is not known if C1q binding will always lead to C1 activation.

In the present study one of the patients with hypocomplementemia showed an inverse relationship between levels of C1r-C1s-C1 IA and C1r-C1s complexes in serial samples (Fig. 2). This may reflect that C1q binding bacterial components or immune complexes with or without C1 activating properties appear during the course of the illness. Similar reasons might be sought to explain the differences in the C1 subcomponent pattern between adults and young children with acute pneumococcal otitis media.

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BACTERIA ASSOCIATED WITH ACUTE OTITIS MEDIA HAVE HIGH C1_q BINDING CAPACITY

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A simple, rapid and sensitive radioimmunologic method for demonstrating C1_q binding to bacteria is described. Various bacteria were shown to bind C1_q without the participation of antibodies. Great differences in C1_q binding levels between different types and strains of *S. pneumoniae* were found. A high uptake of C1_q was observed for many pneumococcal strains of type VI, XIX and XXIII and also for non-typable strains of *H. influenzae* and *B. catarrhalis*. Other bacteria tested, including *H. influenzae* types a-f, demonstrated less capacity to bind C1_q.

Key words: Complement; C1_q-binding substances, pneumococci; haemophilus; branhamella.

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Decreased serum levels of C1_q with concomitant normal or elevated levels of C1r and C1s are found in many patients with otitis media (2, 7). Recently we have reported C1_q binding substances in the sera and middle ear effusions of such patients (7). The nature of these C1_q binding substances have not been recognized but they may represent complexes of antibodies and bacterial antigens. Loos *et al.* (8) have shown that the first component of complement, C1, binds to bacterial lipopolysaccharides (LPS), without the presence of antibodies, and they also demonstrated that intact bacteria such as *E. coli* and *K. pneumoniae* had this capacity (9).

In the present study a method to quantitate the uptake of C1_q by intact bacteria was designed. Bacterial species known to cause acute otitis media in children were investigated regarding their capacity to bind C1_q independently of the presence of antibodies.

MATERIAL AND METHODS

Bacterial Strains and Suspensions

Eighty-seven strains were obtained from clinical specimens sent to the Department of Medical Microbio-

logy, University Hospital, Lund, for bacteriological examination. The bacteria were: 37 strains of *S. pneumoniae* of which 3 were type I; 4 were type III; 4 were type VI; 4 were type XIV; 4 were type XVIII; 13 were type XIX and 5 were type XXIII; 22 strains of *H. influenzae* of which 2 were type a; 7 were type b; 3 were type c; 3 were type d; 3 were type f and 4 were non-typable; *B. catarrhalis*, 7 strains; *E. coli*, 4; *S. aureus*, 4; *S. epidermidis*, 2; *S. saprophyticus*, 2; group A streptococcus, 1; group B streptococci, 2; group C streptococci, 2 and *S. faecalis*, 2 strains.

S. pneumoniae, *H. influenzae* and *E. coli* were identified according to conventional methods. The pneumococcal serotypes were determined by the Quellung reaction of Neufeld (10). *H. influenzae* were typed by slide agglutination. *B. catarrhalis* was identified as described by Kamme (3). The *S. aureus*, *S. epidermidis* and *S. saprophyticus* strains were identified by conventional methods including test for novobiocin resistance (13). The streptococci were serologically grouped (1) and *S. faecalis* tested for tellurite resistance (11).

H. influenzae strains and *B. catarrhalis* strains were obtained from hematin agar plates incubated at 37 °C for 18 hours in 6% CO₂ atmosphere. The other strains were incubated in 20 ml Todd Hewitt broth at 37 °C for 18 hours. The bacteria were washed three times in sucrose veronal-buffer (SVB): 0.27 moles sucrose, 25 mmoles sodium barbital, 0.1 mmol Ca⁺⁺ and 0.5

mmol Mg^{++} per liter, pH 7.2. The bacterial suspensions were adjusted to $A_{540} = 0.70$ by opacity determinations. The suspensions were concentrated ten times by centrifugation before use in the Cl_q binding assay.

Cl_q

Cl_q was purified from fresh human serum according to Volanakis & Stroud (14). The purified Cl_q was labelled with ^{125}I as described by Sobel *et al.* (12) and stored at $-80^{\circ}C$. ^{125}I Cl_q 0.3 mg/ml in 0.1 M phosphate buffer, pH 7.0, was centrifuged at 13,000 g for 5 min in a Beckman microfuge before each experiment.

Estimation of the Capacity of Bacteria to Bind Cl_q

Principally, the procedure described by Sobel *et al.* (12) for binding of Cl_q to sensitized sheep cells (EA), was followed: 200 μ l of SVB, 100 μ l of the bacterial suspension to be tested and 10 μ l of ^{125}I Cl_q was mixed. Two hundred μ l of the mixture was layered on 150 μ l of a 40% sucrose solution in 0.05 M sodium phosphate buffer, pH 7.0, in a 400 μ l polyethylene microfuge tube (Beckman Instruments, Inc., Clinical Instrument Division Fullerton, Calif.). After centrifugation at 13,000 g for 15 min the tube was clamped with a hemostat above the sedimented bacteria and the tip cut off. The radioactivity in the pellet and the supernatant was measured in an LKB-Wallac 1270 Rackgamma gamma radiation counter (A. B. Biotec, Stockholm, Sweden). The Cl_q uptake by the bacterial sediment was calculated as a percentage of the radioactivity of the total Cl_q added.

RESULTS

Methodological Investigation

Incubation temperature. The uptake of ^{125}I labelled Cl_q by different strains of pneumococci and *E. coli* was tested at $0^{\circ}C$, $20^{\circ}C$ and $37^{\circ}C$, maintaining the incubation time at 20 min and the conductance of the buffer at 7 mmho/cm. Increasing the temperature from $0^{\circ}C$ to $20^{\circ}C$ increased the Cl_q uptake by *S. pneumoniae* type XIX from 35 to 72 per cent and by *E. coli* from 22 to 38 per cent. At $37^{\circ}C$ the Cl_q uptake was increased further to 80 and 50 per cent, respectively (Fig. 1). For practical reasons an incubation temperature of $20^{\circ}C$ was used in the following experiments.

Incubation time. The Cl_q uptake at $20^{\circ}C$ was determined after incubation of the reagent mixture for 0, 5, 10, 20, 30, 60 and 120 min, respectively. The conductance of the buffer used was 7 mmho/cm. Ninety per cent of the total amount of Cl_q bound during 120 min incubation was taken up within the first 10 min. This result was obtained with all strains tested (*S. pneumoniae* type VI, XIV, XVIII, XIX and *E. coli*). The capacity of *S. pneumoniae* type XIX to bind Cl_q at various

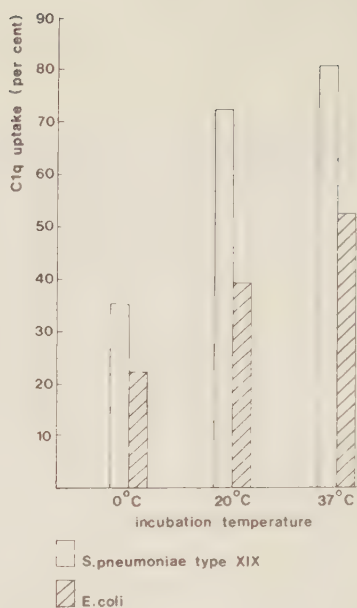


Fig. 1. Binding of ^{125}I Cl_q to *S. pneumoniae* type XIX and to *E. coli* at different incubation temperatures.

incubation times is shown in Fig. 2. An incubation time of 20 min was used in the following studies.

Conductance. The effect of varying the conductance of the SV-buffer used in the experiments was investigated. Cl_q uptake was determined for one strain of each *S. pneumoniae* type VI, XIV, XIX, XXIII and one *E. coli* strain. The highest level of Cl_q binding was obtained between 7 and 9 mmho/cm. Further increase of the conductance diminished the Cl_q uptake on the bacteria, as illustrated with *S. pneumoniae* type XXIII in Fig. 3. In the following experiments a conductance of 7 mmho/cm was used.

Variation in the values for uptake of Cl_q . The uptake of Cl_q was measured for one strain of each *S. pneumoniae* type III and type XIX, cultured in 20 ml Todd Hewitt broth at 5 and 13 different occasions, respectively, using one Cl_q preparation. The Cl_q uptake was expressed as a percentage, mean $\pm$ 1SD and was for type III: 9.9 ± 3.8 and for type XIX: 76.8 ± 4.8 .

When different batches of Cl_q were used, the numerical values for Cl_q uptake differed. For one strain of *S. pneumoniae* (type XIX) Cl_q uptake varied from 59 to 80 per cent. However, the relation between the Cl_q uptake for different bacterial strains was not influenced by the Cl_q preparation used.

In control experiments unlabelled Cl_q was found to inhibit the uptake of radio-labelled Cl_q .

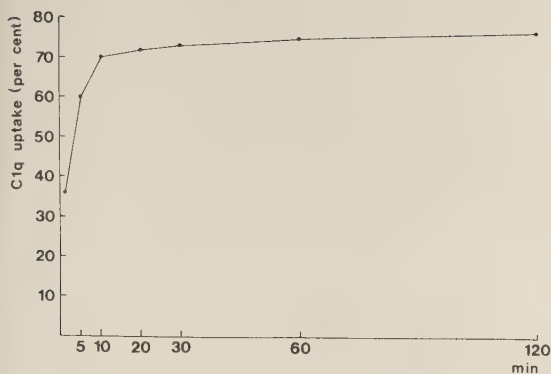


Fig. 2. Kinetic analysis of ^{125}I C1_q uptake by *S. pneumoniae* type XIX.

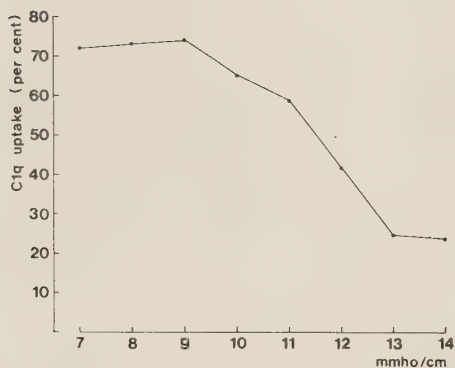


Fig. 3. Binding of ^{125}I C1_q to *S. pneumoniae* type XXIII as a function of the conductance of the buffer.

Binding of C1_q to Different Bacteria

The results of C1_q binding of various types of pneumococci and *H. influenzae* as well as of *B. catarrhalis* and *E. coli* are presented in Fig. 4.

Non-typable *H. influenzae* had the highest C1_q binding capacity varying between 87 and 92 per cent for the four strains tested. *H. influenzae* type a, b, c, d and f demonstrated less C1_q binding and greater variation between different strains (5–55 per cent). All strains of *B. catarrhalis* produced high and constant C1_q binding values (62 to 76 per cent).

The C1_q uptake of the different *S. pneumoniae* types varied. All type III strains tested gave low values (5 to 14 per cent). Marked variation were

noted between different strains of *S. pneumoniae* type I, VI, XIV, XVIII, XIX and XXIII. A total of twenty strains of type VI, XIX and XXIII were tested and eleven of these demonstrated C1_q binding levels greater than 60 per cent.

Four strains of *E. coli* tested demonstrated C1_q uptake of between 31 and 40 per cent.

Streptococcus group A, B, C, G and *S. faecalis* bound C1_q at levels between 27 and 55 per cent. The C1_q uptake for four strains of *S. aureus* was between 34 and 54 per cent. Two strains each of *S. saprophyticus* and *S. epidermidis* tested, produced C1_q binding levels about 20 and 35 per cent, respectively.

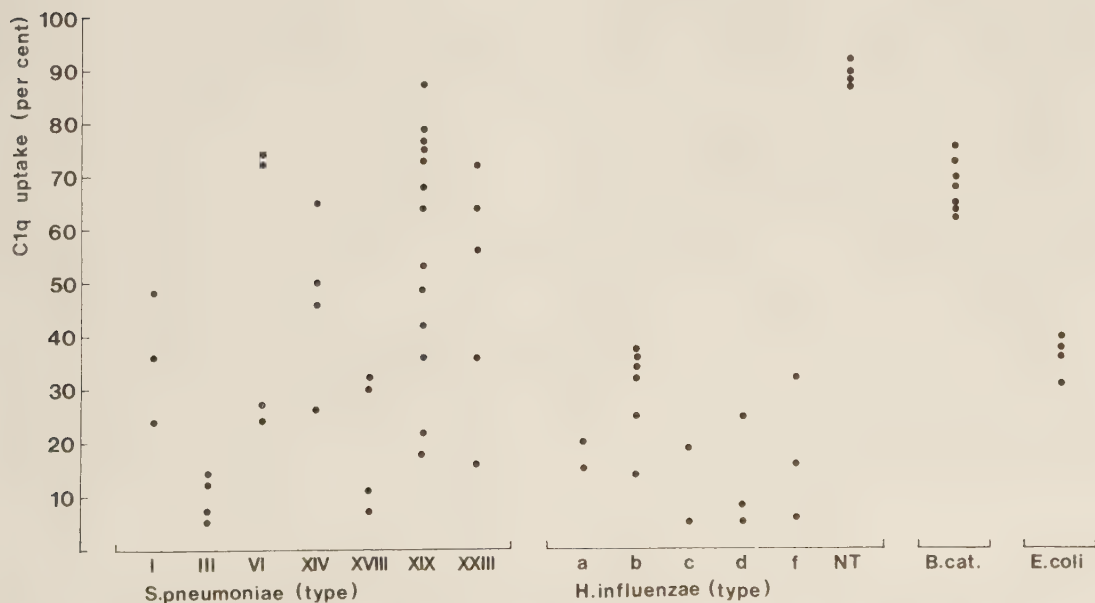


Fig. 4. Binding of ^{125}I C1_q to bacteria at 20 °C, 20 min incubation time and the conductance of the buffer being 7 mmho/cm.

DISCUSSION

The method described here for demonstrating C1_q binding to bacteria is simple, rapid and sensitive. This study presents the capacity of various bacteria to bind C1_q without the participation of antibodies.

Our results concerning C1_q uptake by *E. coli* were in accordance with the findings of Loos *et al.* (9). Furthermore, we found that other bacteria, gram-positive and gram-negative, demonstrated even higher C1_q binding.

In acute purulent otitis media *S. pneumoniae* is the most common causative agent, followed by *H. influenzae* and *B. catarrhalis* (6). Among pneumococci we found a great difference in C1_q binding levels between the different types and strains. A high degree of C1_q uptake was observed for many strains of types VI, XIX and XXIII, which are the serotypes most often isolated in pneumococcal otitis in young children (4). Otitis media caused by *H. influenzae* is in 85 per cent provided for by non-typable strains (5). In this investigation a striking difference in C1_q uptake was demonstrated between *H. influenzae* type a, b, c, d, f and the non-typable strains. The C1_q uptake with typable *H. influenzae* strains varied from 5 to 55 per cent, while non-typable strains produced values of about 90 per cent. A high binding capacity (approximately 70 per cent) was also demonstrated with different strains of *B. catarrhalis*. Thus, bacteria most commonly seen in acute otitis media produced the greatest C1_q binding capacity.

In acute purulent otitis media disorders in the complement system have been demonstrated with decreased serum levels of C1_q and the circulation of abnormal complexes comprising C1 subcomponents, C1r-C1s and C1r-C1s-C1 inactivator (2). An interaction between certain bacteria and C1_q may explain these complement aberrations.

It has been proposed that binding of C1 to bacteria could represent an early defence mechanism against microbial infections (9). However, in acute otitis media it could also be speculated that C1_q binding by bacteria contribute to C1 aberrations which induce an impairment in C activation through the classical pathway, which could lead to defective opsonization of the bacteria.

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COMPLEMENT ACTIVATION BY PNEUMOCOCCI ASSOCIATED WITH ACUTE OTITIS MEDIA

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Prellner, K. Complement activation by pneumococci associated with acute otitis media. Acta path. microbiol. scand. Sect. C, 87: 213-216, 1979.

Pneumococci (types, I, III, VI, XIV, XVIII, XIX and XXIII) associated with acute otitis media were shown to activate complement in normal human serum by the classical as well as by the alternative pathway. In serum incubated with pneumococci classical pathway activation was demonstrated by decreased C4 values and the appearance of C1r-C1s-C1 IA complexes. Pneumococci caused C3 conversion in C2-deficient serum and in serum chelated with Mg^{++} EGTA showing activation of the alternative pathway without participation of the C42 convertase. Complement activation was more efficient when both pathways were intact. This was evident from a more pronounced C3 conversion and a greater reduction of the values for properdin and factor B in non-chelated serum as compared to Mg^{++} EGTA chelated serum.

Key words: Complement activation; pneumococci; acute otitis media.

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Relapsing acute otitis media in children, most often caused by pneumococci (11), is a serious clinical problem. The factors responsible for the relapses are not known.

It is well documented that C3b plays a major role in the opsonisation of pneumococci by normal human serum (9, 20). C3b can be generated by classical pathway activation initiated by antigen-antibody complexes or by activation of the alternative pathway.

Immunoglobulin levels are reported to be within normal levels in children with acute otitis media (1). Prophylactic trials with polyvalent pneumococcal polysaccharide vaccines have shown that the antibody response to some capsular types was suboptimal in children younger than two years of age (6). Aberration of the complement system has been reported in children with relapsing otitis media due to pneumococci (7). Thus, C1q levels were depressed in 60 per cent of patients with relapsing otitis media in combination with normal or elevated levels of C1r and C1s. Increased amounts of

complexes composed of C1 subcomponents, C1r-C1s and C1r-C1s-C1 IA, were found (7). Thus, opsonisation dependent on activation of the classical pathway might be impaired.

The capacity of different pneumococcal types to activate complement has been reported by Fine (3). The present study is concerned with the activation of complement by the pneumococcal types commonly present in relapsing acute otitis media (11).

MATERIALS AND METHODS

Normal human sera from three healthy donors and *serum from a homozygotic C2 deficient adult* were collected and frozen in aliquots at $-80^{\circ}C$ within 3 hours of sampling. Specific pneumococcal polysaccharide antibodies to type I, III, VI, XIV, XVIII, XIX and XXIII, assessed by immunofluorescence (22), were present in low titers in the sera.

Ethyleneglycol tetracetic acid (EGTA) containing magnesium chloride (Mg^{++}) was used as an inhibitor of the classical pathway. In the final experiments, a concentration of 10 mM EGTA and 2.5 mM $MgCl_2$ was used.

Ethylenediamine tetracetic acid (EDTA) in a concentration of 20 mmol/l was used to block the classical and the alternative pathways.

Streptococcus pneumoniae strains of serotypes III, VI, XIV, XVIII, XIX, and XXIII were available from the department. Serotype I, four strains, was supplied by Statens Seruminstitut, Copenhagen, Denmark. Pneumococci were cultivated for 18 hours in Todd-Hewitt broth, washed three times and suspended in phosphate buffered saline (0.12 M NaCl, 0.03 M phosphate), pH 7.2. The suspensions were adjusted to approximately 3×10^9 organisms per ml by density determinations at 540 nm. In some experiments bacteria killed by boiling for 10 minutes were used.

Treatment of sera with pneumococci. Incubation was carried out at 37° C for 60 minutes using viable or heat-killed pneumococci at approximately 5×10^8 organisms per ml serum. Before complement analysis, the bacteria were removed by centrifugation. Normal serum, Mg⁺⁺EGTA chelated serum and C2 deficient serum treated at 37° C for 60 minutes and EDTA chelated serum incubated with pneumococci were used as controls.

Hemolytic complement activity was determined according to Kabat & Mayer (10) and expressed as per cent of hemolytic activity of the relevant control serum. Mg⁺⁺EGTA chelated sera were recalcified (25 mmol Ca⁺⁺ per ml) before analysis.

Quantitations of complement components. The electro-immunoassay (14, 17, 21) was employed for determinations of C1q, C1s, C3, C4, factor B and properdin.

C1 subcomponents complexes. Complexes of C1r-C1s and complexes of C1r-C1s-C1 IA were studied by crossed immunoelectrophoresis (13). C1r-C1s-C1 IA complexes were measured immunochemically according to Laurell *et al.* (16). Pooled normal serum treated with heat aggregated IgG with all C1s antigen present in the C1r-C1s-C1 IA complex was used as a reference. On double determinations the time to time variation was 1.4 per cent (SD) (16).

C3 conversion was assessed by crossed immunoelectrophoresis (4) and evaluated by planimetry. The proportion of converted C3, mainly C3c, was expressed as a percentage of the total area outlined by precipitate. In each assay, the C3 conversion was given as a percentage after correction for the C3 conversion present in the appropriate control.

Statistical significance was assessed by t-test for paired data.

RESULTS

Complement components levels. Incubation of normal serum with pneumococci of the serotypes studied did not change the levels of C1q, C1s, or C3. C4 and factor B values were slightly reduced in non-chelated sera after incubation with the pneumococci but not in sera chelated with Mg⁺⁺EGTA. After incubation with pneumococci the concentration of properdin was low in non-chelated as well as in chelated sera (Table 1). Also in C2-deficient serum the properdin levels fell markedly on incubation with pneumococci. In the non-chelated sera the properdin levels were significantly lower than in the Mg⁺⁺EGTA chelated sera ($p < 0.025$). The values of the complement factors did not differ in experiments using viable or heat-killed bacteria.

All pneumococcal types activated complement which resulted in low residual hemolytic activity. After incubation with pneumococci the residual hemolytic complement activity was reduced to less than 5 per cent of the original value in non-chelated serum and 10–15 per cent in Mg⁺⁺EGTA chelated serum.

C1 subcomponent complexes. Quantitation of C1r-C1s-C1 IA complexes of the normal serum

TABLE 1. Levels of C4, Factor B and Properdin in Normal Non-chelated Serum and in Mg⁺⁺EGTA Chelated Serum after Incubation with Different Pneumococcal Serotypes

Pneumococcal serotypes	C4		Factor B		Properdin	
	non-chelated serum	Mg ⁺⁺ EGTA serum	non-chelated serum	Mg ⁺⁺ EGTA serum	non-chelated serum	Mg ⁺⁺ EGTA serum
I	78	94	93	101	55	63
III	70	99	82	101	31	55
VI	67	89	82	109	48	55
XIV	63	103	76	109	39	55
XVIII	56	99	76	115	55	63
XIX	67	109	70	115	35	50
XXIII	70	99	76	124	48	63

Values given as percentage of non-incubated sera.

TABLE 2. *Per Cent of C3 Conversion Caused by Incubation with Pneumococci of Different Serotypes*

Pneumococcal serotype	Normal non-chelated serum	Mg ⁺⁺ EGTA chelated serum	C2-deficient serum
I	48	41	24
III	60	42	24
VI	53	42	25
XIV	52	41	24
XVIII	53	49	24
XIX	60	41	24
XXIII	54	42	23

The values were obtained by planimetric evaluation. See Materials and Methods.

used as control gave a value of 18 per cent of the reference serum (16).

On incubation of serum with pneumococci of different types, the C₁r-C₁s-C₁ IA complexes increased to values ranging between 30 per cent (type I) and 45 per cent (type XIX) indicating C₁ activation (15). No C₁r-C₁s complexes were observed.

C3 conversion. All the pneumococcal types gave rise to C₃ conversion in non-chelated, Mg⁺⁺ EGTA chelated and C2 deficient serum. The C₃ conversion was more pronounced in non-chelated serum as compared to Mg⁺⁺ EGTA and C2-deficient serum (Table 2).

DISCUSSION

The present investigation showed that serotypes of *Streptococcus pneumoniae* most often found in acute otitis media (I, III, VI, XIV, XVIII, XIX, and XXIII) gave rise to C₃ conversion in serum chelated with Mg⁺⁺ EGTA and in C2-deficient serum. These findings indicate that the strains were capable to activate the alternative pathway without participation of the earlier factors of the classical pathway, C₁, C₄, C₂. Fine (3) studied alternative pathway activation mainly by other pneumococcal types than those pertinent to relapsing otitis media. His data concerning type III and XIV are in agreement with those found in this study. On the other hand, our results gave evidence of alternative pathway activation also by type I pneumococci (four strains tested).

The presence in serum of complexes composed of C₁r, C₁s and C₁ inactivator protein signifies activation of C₁ (15). The formation of C₁r-C₁s-C₁ IA complexes on incubation of normal serum with pneumococci provided evidence for classical pathway activation which was also supported by the decreased C₄ levels. After treatment with pneumo-

cocci the residual hemolytic capacity diminished in non-chelated and in recalcified Mg⁺⁺ EGTA chelated serum indicating marked C activation and consumption by the pneumococci.

Quantitative estimation of factor B gave decreased values in the experiments with non-chelated serum only, and probably indicates additional generation of C₃ convertases when both pathways were intact. It has been shown earlier that C₄ of the classical pathway by conversion of C₃ to C₃b is capable of initiating the C₃b feed back cycle and thus activate factor B (8, 18).

Pneumococci induced a marked decrease of properdin in C2-deficient serum, in non-chelated and in Mg⁺⁺ EGTA chelated serum. Reduction of properdin was most marked in non-chelated serum (Table 1), which might be explained by the amplification of the C₃b feed back cycle by the C₄ convertase. McLean *et al.* (19) have shown that in normal human serum the concentration of properdin was decreased following incubation with antigen-antibody complexes, inulin or zymosan. In C2-deficient serum reduced properdin values were obtained only with zymosan. Thus, the effect of pneumococci on properdin levels seems to be analogous to the effect of zymosan.

Heat killed bacteria were as potent as viable organisms in activating the complement system. This finding indicates that complement activation was most probably not induced by bacterial enzymes.

Complement activation by the classical pathway may be due to antigen-antibody complexes. The serum used contained small amounts of antibodies to capsular pneumococcal antigens. Search for antibodies to other antigens, as C-polysaccharide (CPS) or pneumolysin, was not done. A complex of CPS and C-reactive protein has been reported to activate both the classical and the alternative pathway (2, 12). CRP-CPS complexes are probably

not responsible for complement activation observed in our experiments since the CRP level in the serum used was low (<12 mg per l).

Activation of the alternative pathway by pneumococcal cell wall preparations, which contain teichoic acids and peptidoglycan, have been shown by Winkelstein & Tomasz (23). One or both of these substances may be the pneumococcal constituent responsible for activating the alternative route (5, 23).

The pneumococcal types studied by us were capable of activating complement by both classical and alternative pathways, and the conversion of C3 was most pronounced when both pathways were functional. Earlier studies (7) showed impairment of the classical pathway in relapsing pneumococcal otitis media. It is possible that generation of C3b by the alternative pathway alone might not be sufficient for optimal opsonisation and elimination of the pneumococci and therefore be a contributing factor to relapses observed in acute otitis media.

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PURIFICATION OF C-REACTIVE PROTEIN ON DEAE-CELLULOSE BY A SIMPLE TWO-STEP PROCEDURE UTILIZING THE CALCIUM-DEPENDENCY OF THE PROTEIN

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SUMMARY

A simple procedure for isolating C-reactive protein is presented. The method consists of two chromatographic separations on diethylaminoethyl (DEAE)-cellulose columns and utilizes the difference between the binding of C-reactive protein to DEAE in the presence and absence of calcium. Electrophoretically pure C-reactive protein can be recovered from serum or ascitic fluid with a yield of approx. 50-60% within one day.

INTRODUCTION

C-Reactive protein is built up of six identical, non-covalently bound subunits and has a molecular weight of 120 000 [1]. The protein binds calcium [2]. When saturated with calcium C-reactive protein migrates electrophoretically in agarose gel like a slow γ -globulin. If calcium is omitted from the buffer, the protein migrates like a β_2 -globulin [3]. Several methods are available for purifying C-reactive protein, some including separation on DEAE-cellulose [4, 5]. A method described by Ganrot and Kindmark [3] utilized the ability of C-reactive protein to adsorb to BaSO₄ and to agar gel in the presence of calcium. Mortensen et al [6] in 1975 purified C-reactive protein by affinity chromatography based on the calcium-dependent binding of C-reactive protein to the C-polysaccharide of pneumococci.

We found that the binding strength of C-reactive protein to DEAE-cellulose varied with the presence or absence of calcium ions. We utilized this difference in the development of a simple two-step procedure for preparing C-reactive protein.

MATERIALS

For the chromatographic procedures, Whatman DEAE 32 Microgranular was used.

The following buffers were used in the chromatographic separations: Buffer A,

Tris 40 mmol/l, pH 7.4, containing calcium 5 mmol/l. The conductance was adjusted with NaCl to $19 \text{ m}\Omega^{-1}/\text{cm}$. Buffer B, Tris 40 mmol/l, pH 7.4, containing Na_2EDTA 5 mmol/l. The conductance was set at $24 \text{ m}\Omega^{-1}/\text{cm}$ by addition of NaCl. Conductance was measured at 22°C with a Kemotron Tetramatic equipment.

Agarose was purchased from Miles Seravac Ltd., U.K. In the electrophoretic experiments two buffer systems were used: 75 mmol/l barbital/HCl, pH 8.6, containing calcium 2 mmol/l, and the same buffer in which calcium was replaced by Na_2EDTA 2 mmol/l.

Monospecific antiserum against C-reactive protein was raised by immunizing rabbits with repeated subcutaneous injection of highly purified C-reactive protein.

METHODS

Electrophoresis in agarose gel was performed according to Johansson [7]. Immuno-electrophoresis was done as described by Scheidegger [8]. The electro-immunoassay according to Laurell [9] was used for detecting and measuring C-reactive protein during the purification procedure. The electroimmunoassay was run for 6 h at a potential of 20 V/cm in barbital buffer containing Na_2EDTA .

RESULTS

To ascertain the elution pattern of C-reactive protein from DEAE cellulose and its variation with the presence and absence of calcium, two column chromatographic experiments were done. In the first, 10 ml of ascitic fluid containing 100 mg C-reactive protein/l was applied to a DEAE column equilibrated with Tris 40 mmol/l, pH 7.4, containing calcium at a final concentration of 5 mmol/l. The conductance was adjusted with NaCl to $6 \text{ m}\Omega^{-1}/\text{cm}$. Before use, the ascitic fluid was dialysed against the starting buffer for 8 h. The proteins were eluted with a NaCl gradient of 6 to $40 \text{ m}\Omega^{-1}/\text{cm}$. C-Reactive protein was eluted at $12 \text{ m}\Omega^{-1}/\text{cm}$ (Fig. 1A). In the second experiment the buffer was modified by replacing calcium with Na_2EDTA at a final concentration of 5 mmol/l and under these conditions C-reactive protein was

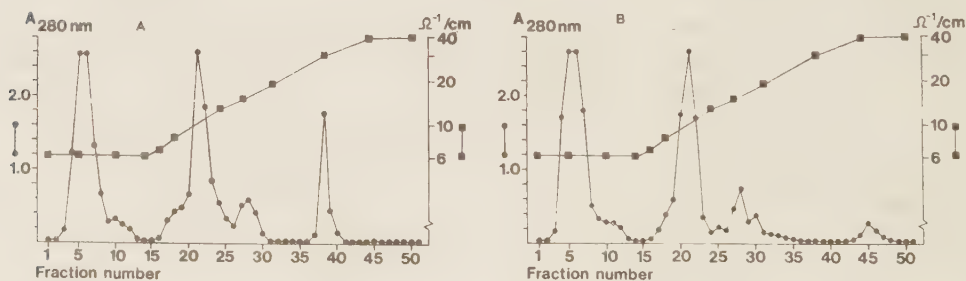


Fig. 1. A. Gradient-elution chromatogram of ascitic fluid on DEAE-cellulose in Tris 40 mmol/l containing calcium 5 mmol/l, pH 7.4. A NaCl-gradient was applied from 6 to $40 \text{ m}\Omega^{-1}/\text{cm}$. The volume of the fractions was 6 ml. B. Gradient-elution chromatogram of ascitic fluid on DEAE-cellulose in Tris 40 mmol/l containing Na_2EDTA 5 mmol/l, pH 7.4. A NaCl-gradient was applied from 6 to $40 \text{ m}\Omega^{-1}/\text{cm}$. The volume of the fractions was 6 ml.

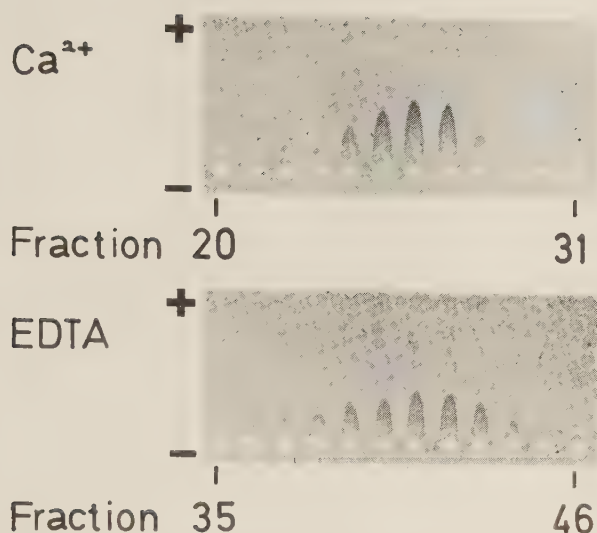


Fig. 2. Electroimmunoassay, in which a specific antiserum against C-reactive protein was used, showing the appearance of C-reactive protein in the fractions from DEAE-cellulose chromatography of ascitic fluid in the presence and absence of calcium (Fig. 1, A and B).

eluted at $27 \text{ m}\Omega^{-1}/\text{cm}$ (Fig. 1B). The fractions obtained from the two columns were screened with electroimmunoassay, in which a specific antiserum against C-reactive protein was used. Fig. 2 shows the appearance of C-reactive protein.

Based on these observations, the following procedure for the purification of C-reactive protein was used. Pooled human serum with increased levels of C-reactive protein ($>100 \text{ mg/l}$) or ascitic fluid from patients with advanced neoplastic diseases served as a source of C-reactive protein for the purification. Before application of serum or ascitic fluid to the first column calcium was added to a final concentration of 5 mmol/l , and the conductance was raised to $19 \text{ m}\Omega^{-1}/\text{cm}$ by the addition of NaCl.

Column 1. The sample was applied to a DEAE-cellulose column ($2.5 \times 20 \text{ cm}$) previously equilibrated with buffer A (see Materials). At this conductance ($19 \text{ m}\Omega^{-1}/\text{cm}$) about 80% of the plasma proteins together with C-reactive protein were not bound to the cellulose and were washed out with 3 to 4 column volumes of buffer A.

Before application to the second column Na_2EDTA was added to the break through protein fraction to a final concentration of 20 mmol/l and the conductance was increased to $24 \text{ m}\Omega^{-1}/\text{cm}$ by the addition of NaCl.

Column 2. The protein fraction from column 1 was applied to a second DEAE-cellulose column ($2.5 \times 20 \text{ cm}$) previously equilibrated with buffer B (see Materials). Because of a higher affinity to DEAE in the absence of calcium, C-reactive protein was bound to the column despite a higher conductance ($24 \text{ m}\Omega^{-1}/\text{cm}$). Proteins other than C-reactive protein were eluted by extensive washing with buffer B. The conductance of buffer B was adjusted to $35 \text{ m}\Omega^{-1}/\text{cm}$ by the addition of NaCl and C-reactive protein was regularly eluted at a conductance of $27 \text{ m}\Omega^{-1}/\text{cm}$. On agarose gel electrophoresis the protein appeared as a single band (Fig. 3). The C-reactive protein preparation was also analysed on immunoelectrophoresis with an antiserum against whole human serum at a concentration of 3 g/l and no contami-

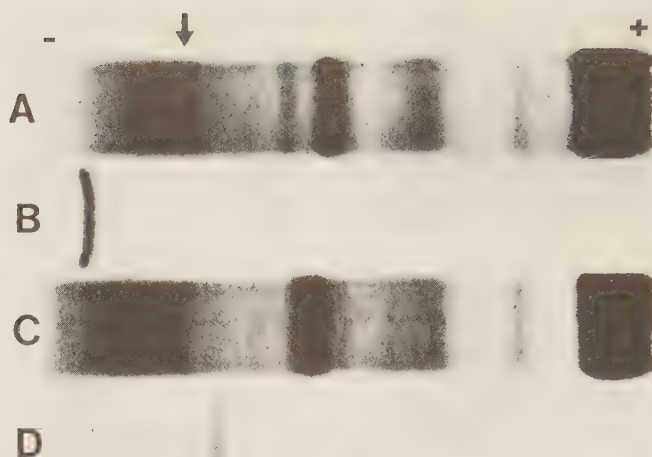


Fig. 3. Agarose gel electrophoresis of normal human serum and the C-reactive protein preparation in barbital/HCl buffer, pH 8.6. A, Normal human serum in the presence of Ca^{2+} 2 mmol/l; B, The C-reactive protein preparation in the presence of Ca^{2+} 2 mmol/l; C, Normal human serum in the presence of Na_2EDTA 2 mmol/l; D, The C-reactive protein preparation in the presence of Na_2EDTA 2 mmol/l.

nating proteins could be detected. However, if the washing procedure of column 2 was insufficient, traces of albumin and a non-identified β_2 -globulin were seen. The yield of the method was calculated with electroimmunoassay and was found to vary between 50 and 60%.

DISCUSSION

The method described for preparing C-reactive protein differs from other methods [4, 5], in which DEAE column chromatography is used in that advantage is taken of the variation of the binding of C-reactive protein to DEAE-cellulose with the presence and absence of calcium. If calcium is present during the chromatography, C-reactive protein is only weakly bound to DEAE and is eluted at a conductance of $12 \text{ m}\Omega^{-1}/\text{cm}$. However, if calcium is removed by chelation with Na_2EDTA , C-reactive protein is firmly bound and is eluted at $27 \text{ m}\Omega^{-1}/\text{cm}$. The different chromatographic behaviour of C-reactive protein is explained by its strong interaction with calcium.

The yield of the method varies between 50 and 60%, which is less than for other methods described [1]. However, this is compensated by the quickness and simplicity of the method. Furthermore, as only small amounts of protein are bound to the cellulose under conditions used in the two chromatographic steps, large amounts of starting material can be applied to relatively small columns without exceeding the binding capacity of the DEAE and thereby shortening the elution time for each step.

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Cl_q BINDING AND COMPLEMENT ACTIVATION BY CAPSULAR AND CELL WALL
COMPONENTS OF S.PNEUMONIAE_TYPE XIX

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SUMMARY

Cell wall components (purified cell walls, teichoic acid and residual cell walls) from S. pneumoniae type XIX showed antibody independent C1q binding capacity, as assessed by C1q deviation test, with teichoic acid being the most efficient. Specific capsular substance did not bind C1q. All substances tested produced C1 activation in normal human serum, but not in hypo- γ -globulinemic serum. Thus, teichoic acid showed high C1q binding capacity but did not activate C1 in the absence of antibodies.

Teichoic acid was an effective activator of the alternative pathway. Specific capsular substance did not activate the alternative pathway in C1q deficient serum or in Mg^{2+} -EGTA chelated normal serum.

Key words: Complement activation, C1q binding, Otitis media, Pneumococci, Teichoic acid

INTRODUCTION

Opsonisation and phagocytosis of pneumococci are essentially dependent on C3b bound to the bacterial cell (36). C3b can be generated either by activation of the classical or the alternative pathway of complement.

Activation of the alternative pathway occurs on incubation of normal human serum with various Gram-negative and Gram-positive bacteria (5, 7, 8, 11, 24, 31, 34). For Gram-negative bacteria the responsible component was the cell wall endotoxic lipopolysaccharide (4, 6, 13, 23), but the component of Gram-positive bacteria responsible for activation of the alternative pathway has not been identified with certainty. The peptidoglycan of staphylococci and of group A streptococci has been demonstrated to be the most active component (12, 33, 35) and for pneumococci teichoic acid is a stronger activator of the alternative pathway than is peptidoglycan (37).

Disorders involving the classical pathway have been reported in children with recurrent pneumococcal otitis media. Decreased amounts of C1q together with increased amounts of complexes composed of C1 subcomponents were constant findings (15).

In vitro studies have shown that some bacteria associated with acute otitis media such as pneumococci, H. influenzae and B. catarrhalis, have high capacity to bind C1q, also in the absence of specific antibodies (25). C1q binding does not always lead to C1 activation which has been demonstrated for polynucleotides (19, 30).

The purpose of the present investigation was threefold, namely, to assess the capacity of pneumococcal wall components and specific capsular polysaccharide to bind C1q in the absence of antibodies, to find out whether C1q binding leads to activation of C1 and to identify the bacterial subcomponent responsible for activation of the alternative pathway.

MATERIALS AND METHODS

Preparation of pneumococcal cell walls. The pneumococcal cell walls were purified largely according to Mosser & Tomasz (1970). *Pneumococcus* type 19, passed three times aerobically on blood agar plates, was grown in Todd-Hewitt broth at 37°C for 18 hours, heated at 70°C for 15 min, washed four times in 0.15 M saline and disrupted by sonication for 60 min. The crude cell walls were isolated by centrifugation, washed twice in 0.15 NaCl and treated for four hours at 37°C with DNase and RNase, during constant stirring, washed three times in 0.15 M saline, centrifuged at 25,000 g and suspended in 0.15 M NaCl and lyophilized.

Extraction of teichoic acid from pneumococcal cell walls. Teichoic acids were extracted from pneumococcal cell wall preparation with hot formamide as described by Fuller (9). The soluble teichoic acids in the supernatant were dialysed extensively against 0.15 M NaCl and lyophilized. The insoluble cell wall components containing the peptidoglycan were washed in saline and lyophilized.

Type specific capsular antigen from pneumococcus 19 was obtained from Merck, Sharp and Dohme Research Laboratories, West Point, Pa.

C-reactive protein (CRP) was purified from pooled human serum or from ascitic fluid (16).

Normal human serum (NHS) from three healthy donors and serum from a C1q deficient child referred to us by Dr. Mikuka, Belgrade, were frozen in aliquots at -80°C within 3 hours of sampling.

Hypogammaglobulinemic serum containing less than 0.3 g per l of IgG and with levels of IgM and IgA of less than 0.01 and 0.001 g per l, respectively, was used in the study. This serum showed a normal hemolytic pattern in a hemolysis in gel assay (32). C1s, C4 and C3 were within normal ranges. C1q was reduced (49 per cent of normal standard), and large amounts of proenzymes C1r-C1s complexes were present, as has been described in hypogammaglobulinemia (18). In the experiments, the C1q level was restored by addition of purified C1q. This resulted in the disappearance of the C1r-C1s complexes and the formation of native C1, as shown by crossed immunoelectrophoresis (18) (fig. 1).

Ethylene glycol tetracetic acid (EGTA) containing magnesium chloride (Mg^{2+}) in a concentration of 10 mM EGTA and 2.5 mM $MgCl_2$, was used as an inhibitor of the classical pathway,

Incubation of sera with pneumococcal cell wall components and specific capsular antigen was carried out at 37°C for 30 min.

Quantification of C1r-C1s-C1 inactivator complexes (C1r-C1s-C1 1A) was done immunochemically, with the use of a reference prepared as described by Laurell et al. (1979). The variation of double determinations made on different occasions was 1.4 per cent (SD).

C3 conversion was assessed by crossed immunoelectrophoresis (10) and evaluated by planimetry. The proportion of converted C3, mainly C3c, was expressed as percentage of the total area outlined by the precipitate. C3 conversion was given as the percentage after correction for C3 conversion in the appropriate control

A

B

C

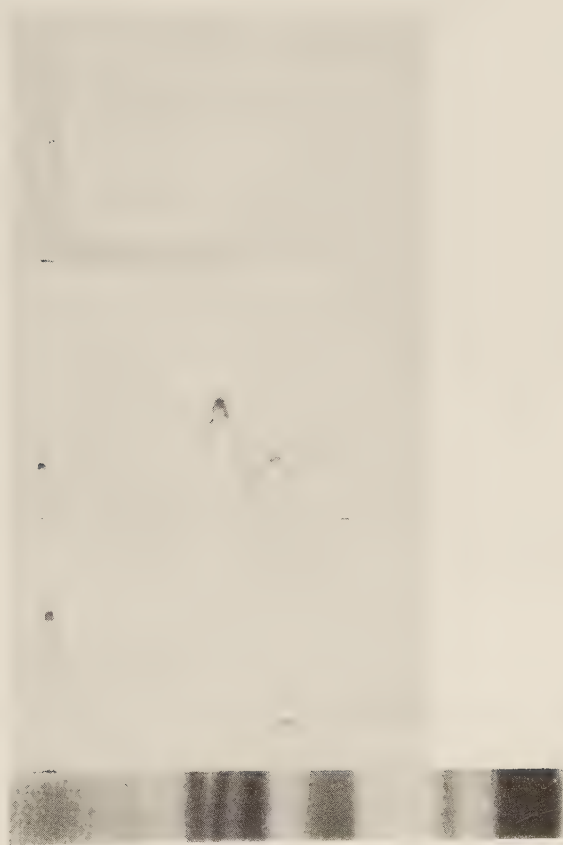


Fig. 1.

experiment.

The C1q binding capacity of the bacterial cell constituents was examined using the principles of the C1q deviation test, C1q DT (30), and of the C1q binding assay, C1q BA (39). To the test systems of 50 μ l veronal buffered saline (VBS, prepared by standard methods) (27) containing human serum albumin (HSA) in a concentration of 50 mg per ml, was added varying amounts of the different bacterial cell constituents and investigated as described earlier (30, 39). Values in the C1q DT were expressed as percentages of inhibition of C1q uptake by IgG-coated sheep erythrocytes. Heat aggregated IgG added to VBS with HSA was used for calibration of the test systems, and quantities between 10 and 2000 μ g per ml buffer could be estimated.

RESULTS

Binding of C1q to different pneumococcal polysaccharides

In the C1q DT teichoic acid showed the highest C1q binding capacity. Thus, the C1q uptake by sensitized sheep erythrocytes was inhibited to 74 per cent, by a concentration of 10^3 μg per ml of teichoic acid. The corresponding values for purified pneumococcal cell wall and for residual cell wall preparations were 56 and 34 per cent, respectively. The pneumococcal type specific capsular polysaccharide did not inhibit the C1q uptake in the C1q DT (fig. 2).

No C1q binding capacity was found for any of the pneumococcal polysaccharides investigated with the C1q BA.

C $\bar{\text{T}}\text{r}$ -C $\bar{\text{T}}\text{s}$ -C $\bar{\text{T}}$ inactivator complexes

The amounts of complexes composed of C $\bar{\text{T}}\text{r}$ -C $\bar{\text{T}}\text{s}$ and C $\bar{\text{T}}$ IA that formed in normal human serum on incubation with various concentrations of pneumococcal polysaccharides are shown in fig. 3. Repeated estimation of the level of C $\bar{\text{T}}\text{r}$ -C $\bar{\text{T}}\text{s}$ -C $\bar{\text{T}}$ IA complexes in the NHS control incubated for the same time with VBS gave values between 18 and 20 per cent. With concentrations increasing up to 10^3 μg per ml of purified pneumococcal cell walls, teichoic acid or residual cell walls the amount of C $\bar{\text{T}}\text{r}$ -C $\bar{\text{T}}\text{s}$ -C $\bar{\text{T}}$ IA complexes steadily increased, equally in all three preparations, to levels of about 50 per cent. Specific capsular polysaccharide was more active than the other preparations tested. At 10 μg per ml of specific capsular substance the concentration of C $\bar{\text{T}}\text{r}$ -C $\bar{\text{T}}\text{s}$ -C $\bar{\text{T}}$ IA was 45 per cent and a peak value of 52 per cent at 10^2 μg per ml of

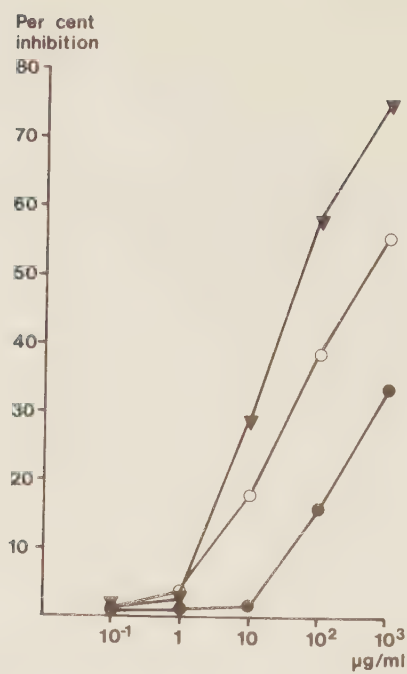


Fig. 2.

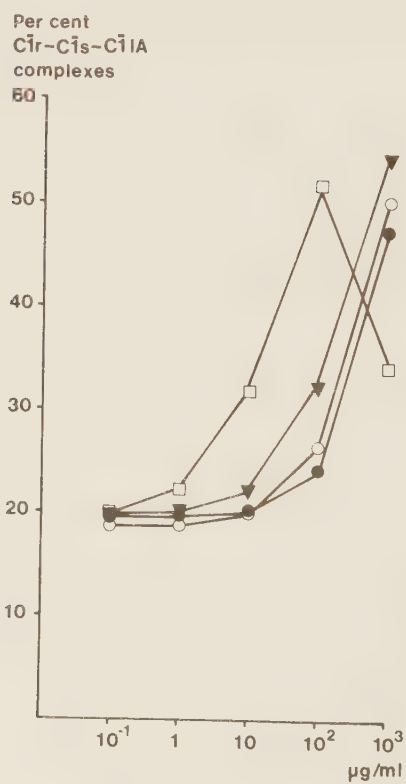


Fig. 3.

specific capsular substance. At 10^3 μg per ml, the value for C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ -C $\bar{\text{I}}$ IA complex level fell. On incubation with teichoic acid or any of the other pneumococcal component preparations C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ -C $\bar{\text{I}}$ IA complexes were not formed in C1q restituted hypo- γ -globulinemic serum. The capacity for C $\bar{\text{I}}$ activation in the C1q restituted hypo- γ -globulinemic serum was assessed by adding CRP-teichoic acid complexes. CRP-teichoic acid complexes were obtained by incubating CRP in a concentration of 50 μg per ml with teichoic acid in a concentration of 250 μg per ml, and the complex formation with CRP was demonstrated by crossed immunoelectrophoresis with anti-CRP in the gel. On addition of CRP-teichoic acid complexes to the hypo- γ -globulinemic serum, C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ -C $\bar{\text{I}}$ IA complexes increased from 24 to 37 per cent (table I).

C3 conversion

In non-chelated serum purified pneumococcal cell walls, teichoic acid, residual cell walls and specific capsular polysaccharide gave rise to C3 conversion, varying between 45 per cent for residual cell walls and 56 per cent for the specific capsular substance (fig. 4 a-d). In normal human serum specific capsular polysaccharide gave more pronounced C3 conversion at concentrations lower than with the different cell wall preparations. Maximal C3 conversion was observed at concentrations of 10^2 μg per ml of specific capsular polysaccharide.

In Mg^{2+} -EGTA chelated serum, all pneumococcal preparations except the specific capsular polysaccharide caused C3 cleavage. The percentage of C3 conversion by the cell wall preparations in chelated serum varied with 17 per cent by residual cell walls in a concentration of 10^3 μg per ml and 25 per cent by teichoic acid.

Table I. Quantity of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ inactivator complexes relative to that in a reference serum, obtained after incubation in hypo- γ -globulinemic serum incubated at 37°C for 30 min with teichoic acid at 250 μ g per ml, CRP at 50 μ g per ml and with complexes of CRP and teichoic acid (see results).

Incubation mixture	Percentage of C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes
hypo- γ -globulinemic serum	24
hypo- γ -globulinemic serum and teichoic acid	26
hypo- γ -globulinemic serum and CRP	28
hypo- γ -globulinemic serum and CRP-teichoic acid complexes	37

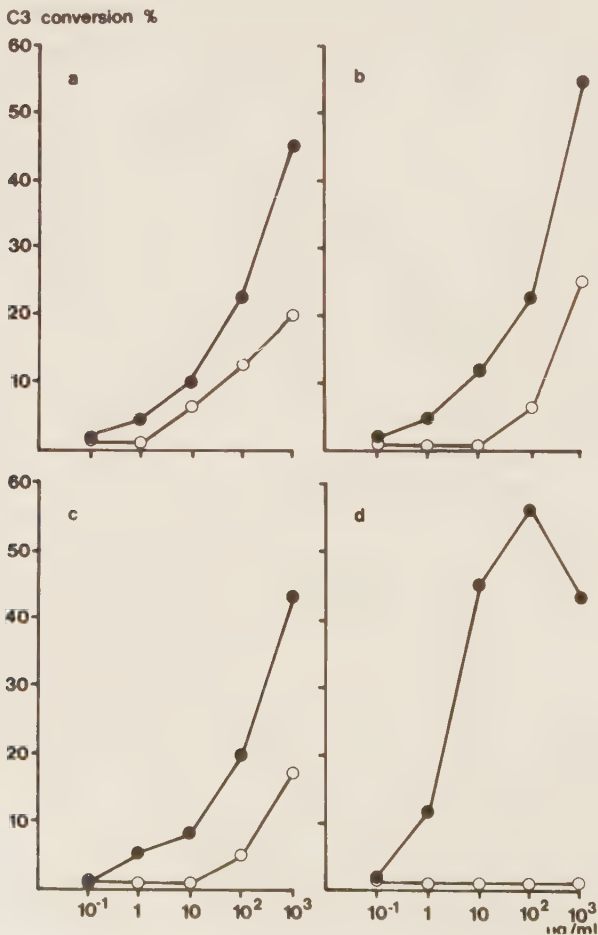


Fig. 4.

In C1q restituted hypo- γ -globulinemic serum and in C1q-deficient serum C3 was converted to the same degree as in chelated serum by pneumococcal cell walls, teichoic acid and residual cell walls. Specific capsular substance produced no C3 conversion in the hypo- γ -globulinemic serum or in C1q-deficient serum.

DISCUSSION

As shown (33, 35) it is cell wall peptidoglycan from staphylococci, rather than teichoic acid, that is responsible for the activation of human complement by the alternative pathway. Greenblatt et al. (1978) also claimed that peptidoglycan from group A hemolytic streptococci is the most active cell wall component of this species activating the alternative pathway. Winkelstein and Tomasz (1978), on the other hand, using C4 deficient quineapig serum, found teichoic acid and not peptidoglycan from pneumococci to be the most effective activator of the alternative pathway. Our findings of alternative pathway activation by pneumococcal teichoic acid in hypo- γ -globulinemic, C1q-deficient ant Mg^{2+} -EGTA chelated human sera are in agreement with the findings of Winkelstein and Tomasz, 1978.

Activation of the C system was also induced by incubation of normal non-chelated human sera with teichoic acid. It is known that cross-reacting antibodies to teichoic acids of various bacteria are common in sera from healthy human individuals (3, 38). The C1 activation appearing in normal serum on incubation with teichoic acid could therefore be explained by teichoic acid-antibody complexes. The C activation occurring on incubation of NHS with specific capsular polysaccharide and residual cell wall component, consisting mainly of peptidoglycan (17), was probably also due to the presence of specific antibodies to these bacterial components (28, 29).

Formation of C1r-C1s-C1 IA complexes was decreased when the dose

of specific capsular polysaccharide was increased from 10^2 to 10^3 μg per ml of normal serum (fig. 3). Immunaggregates formed in antigenic excess are less capable of fixing C1q and thereby activating C1 (2, 22), which might explain this finding.

In a previous study it was shown that pneumococcal types associated with acute otitis media bind C1q also in the absence of antibodies (25). In the present investigation teichoic acid from pneumococcus type XIX, proved the most effective C1q binding component.

C1r-C1s-C1 inactivator complexes are formed on activation of C1 by various well-known C1 activators (19). Such complexes did not appear on incubation of teichoic acid with C1q reconstituted hypo- γ -globulinemic serum, thus indicating that the binding of C1q to teichoic acid does not lead to activation of C1 in the absence of antibodies. Binding of C1q without C1 activation has not convincingly been demonstrated for bacterial polysaccharides earlier, but apparently occurs with certain polynucleotides (19, 30).

Classical pathway activation, which is mediated by antigen-antibody complexes, is known to occur in adults with pneumococcal pneumonia or meningitis (26). Children, below two years of age are known to have relatively lower content of pneumococcal antibodies than adults (1, 14). If especially the IgG antibody response is insufficient, the clearance of pneumococci depends on the presence of IgM antibodies together with an intact complement system. It might, thus, be speculated that C1q consumption by teichoic acid can interfere with classical pathway activation and inhibit the formation of opsonic C3b and

thereby help to explain the relapses of pneumococcal otitis media seen in children.

LEGENDS TO FIGURES

Figure 1

Crossed immunoelectrophoresis of Cls in serum.

A: Normal serum incubated at 37⁰ C for 30 minutes.

B: Hypogammaglobulinemic serum (see Materials and methods) incubated at 37⁰ C for 30 minutes.

C: Hypogammaglobulinemic serum from B, restituted with Clq to the corresponding Cls level, incubated at 37⁰ C for 30 minutes.

Figure 2

Clq-binding activity for increasing concentrations (log scale) of three different pneumococcal cell wall preparations: ○—○ purified pneumococcal cell walls; ▼—▼ teichoic acids extracted with hot formamide; ●—● residual cell walls after formamide extraction. Values are given as percentages of inhibition in a modified Clq-deviation test (see Materials and methods).

Figure 3

Cl activation induced in normal human serum incubated at 37⁰ C for 30 minutes with increasing quantities (log scale) of different pneumococcal components: ○—○ purified pneumococcal cell walls; ▼—▼ teichoic acids extracted with hot formamide; ●—● residual cell walls after formamide extraction; □—□ type specific capsular polysaccharides. Cl activation demonstrated by formation of C1r-C1s-C1 IA complexes and values are given as percentages of a reference serum (see Materials and methods).

Figure 4

C3 conversion in normal serum (●—●) and in Mg²⁺EGTA-chelated serum (○—○) incubated at 37⁰ C for 30 minutes with increasing quantities (log scale) of four different pneumococcal components: a. purified pneumococcal cell walls; b. teichoic acids; c. residual cell walls; d. type specific capsular polysaccharides. Values are given as percentages calculated from planimetric evaluation.

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